EFFECT OF LYSOSOMAL CHOLESTEROL ACCUMULATION ON LYSOSOMAL AND VACUOLAR-ATPASE ACTIVITY

By

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Dissertation
Submitted to the Faculty of the
Graduate School of Vanderbilt University
in partial fulfillment of the requirement
for the degree of

DOCTOR OF PHILOSOPHY
in
Pathology
December, 2007
Nashville, Tennessee

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To my wonderful and supportive family: Stacey, Mom, Dad, RuthAnne, Mema, Papa, Jett, and Savannah

ACKNOWLEDGEMENTS

I would first like to thank my mentor, Jay Jerome. Jay has been a great mentor as well as friend. He has helped steer my enthusiasm for science and kept me focused on the tasks at hand as well. I will forever be grateful for his guidance as a scientist.

I would also like to thank the members of my dissertation committee. Sergio Fazio, Anne Kenworthy, Richard Hoover, Jason Morrow, and Larry Swift have provided excellent guidance and suggestions over the past several years.

I would also like to thank the people in the Jerome Lab and Pathology department past and present. I particular want to thank Jody Ullery and Evelyn Griffin. Jody is a graduate student who I was able to bounce ideas off of and able to work through tough experiments with. Evelyn was a technician in the lab that help guide me when I came into the lab and gave immeasurable assistance in starting my experimental path. I would also like to acknowledge the laboratory of Paul Bock. Without their help many of my experiments would have never taken shape. Additionally, I must thank my funding sources including the Vascular Biology Training Grant and the Pathology Department.

Outside of lab I would like to thank my family. My wonderful wife, Stacey, has been through it all and has been understanding the entire way. I would next like to thank my parents, Al and Deborah. My dad being in science as well has provided assistance and guidance throughout my life. I would also like to thank my sister, RuthAnne, who has always been a confidant and there when I need someone to talk to. Last, but not least I would like to thank Stacey's wonderful family for being there when we need anything over the past several years and it is them that have made Stacey the person she is.

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LIST OF ABBREVIATIONS

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

Acetylated LDL .....ac-LDL

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

Aggregated LDL .....agg-LDL

Apolipoprotein .....apo

ATP binding cassette transporter .....ABC

Cholesteryl ester.....CE

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

Cholesterol Methyl Ether.....CME

Electron spin resonance .....ESR

Free cholesterol.....FC

Gas chromatography .....GC

High density lipoprotein .....HDL

Low density lipoprotein .....LDL

Lysosomal acid lipase.....LAL

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

Lysosomal storage disorders.....LSD

methyl-β-cyclodextrin.....MβCD

Neutral cholesteryl ester hydrolase.....NCEH

Niemann Pick type C .....NPC

Oxidized LDL .....ox-LDL

Regulator of v-ATPase assembly.....RAVE

Reverse cholesterol transport.....RCT

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

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

Site-1 protease.....S1P

Site-2 Protease S2P

Sterol regulatory element binding proteins.....SREBP

SREBP cleavage-activating protein.....SCAP

Transmission electron microscopy .....TEM

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

CHAPTER I

INTRODUCTION

Objective

Atherosclerosis and its clinical complications are a major cause of mortality in developed countries, yet much remains to be learned concerning factors influencing disease initiation and progression. The hallmark of the disease is the presence of macrophages that continually engulf lipid and develop into macrophage foam cells. During early stages of atherosclerosis the lipid that is engulfed is primarily stored as cytoplasmic droplets. However, as the lesion progresses, the lipid storage shifts to the lysosome. The lysosomal accumulation of lipid led de Duve and colleagues to suggest atherosclerosis was a lysosomal storage disorder (Peters et al., 1972; de Duve, 1974; Peters and de Duve, 1974). To date the exact mechanism of this lysosomal accumulation has not been identified. Furthermore, it has been demonstrated that there is an excess of free cholesterol (FC) in the advanced lesion (Li et al., 2005), which is inconsistent with cytoplasmic storage. Moreover, the excess FC could potentially activate macrophage cell death (Zhou et al., 2005). Studies in our lab and others, using macrophages in tissue culture, indicated that after a brief period of lipid uptake the lysosomes of the macrophages fail to hydrolyze the cholesterol ester (CE) on modified lipid particles (Jerome et al., 1998; Yancey and Jerome, 1998; Dhaliwal and Steinbrecher, 2000; Yancey and Jerome, 2001). Therefore, we hypothesized that lysosomal lipid accumulation altered the lysosomal environment such that the lysosomes were no longer able to hydrolyze lipid particles or degrade proteins causing them to be sequestered in the lysosomes. The research presented in this dissertation focuses on understanding the

mechanisms for this lysosomal lipid sequestration by exploring the effect of free cholesterol on lysosomal function and activity of the vacuolar ATPase.

Pathogenesis of atherosclerosis

Cardiovascular disease is the leading cause of death in the western world, accounting for more deaths than the next two causes combined (AHA, 2004). Atherosclerosis, the leading single cause of cardiovascular disease, is a very insidious disease starting during the teenage years, but not exhibiting clinical complications until the fourth or fifth decade of life (Shoen and Coltran, 1999). This indicates that there may be a time dependent component to atherosclerosis; however, atherosclerosis lesion development is more closely related to circulating lipoprotein levels. This has lead Williams and Tabas to develop the "response to retention" hypothesis, which states that lipoproteins, mainly low density lipoproteins (LDL), become trapped in the arterial intimal space and become modified (Williams and Tabas, 1995; Williams and Tabas, 2005). It is this retention-induced modification that leads to the recruitment of macrophages and uptake of modified lipid particles, which are key components of the development of macrophage foam cells. Foam cells are macrophages which have entered the artery wall and engulfed large quantities of lipid. The accumulation of lipid leads to large lipid swollen vacuoles, which give them a foamy appearance. The presence of macrophage foam cells is the hallmark of an atherosclerotic lesion. The further recruitment of macrophages and their development into additional foam cells eventually lead to the formation of clinically significant lesions. Moreover, the retention hypothesis has been refined to include the role that inflammation plays in the development of

clinically significant lesions (Ross, 1999). The inflammatory component is due not only to the recruitment of macrophages, but also the recruitment of other immune cells, such as T-cells and the production of inflammatory cytokines by cells within the lesion (reviewed by (Stoll and Bendszus, 2006)). As the lesion advances, smooth muscle cells are recruited from the arterial media into the developing lesion. Upon recruitment, the smooth muscle cells begin to secrete matrix proteins which can help stabilize the lesion (Jerome and Lewis, 1985). Clinically significant lesions often have a high lipid to matrix ratio and a thin fibrous cap. These lesions are prone to rupture, resulting in hemorrhage and thrombosis leading to myocardial infarction or stroke.

One basic mechanism for the development of the atherosclerotic lesion is the entrapment of LDL within the arterial intima leading to the modification of the LDL particles (Brown and Goldstein, 1976; Brown et al., 1979). Modification of LDL is important because it allows macrophages to take up these particles through unregulated scavenger receptor pathways and not through the highly regulated LDL receptor pathway. Kruth *et al.* have shown that there is an accumulation of FC in the subendothelial space prior to morphological changes in the vessel (Kruth, 1985). In addition to the accumulation of cholesterol, atherosclerotic lesions develop at sites in the artery that have turbulent flow characteristics, such as branch points and areas of high stress (Jerome and Lewis, 1984; Shoen and Coltran, 1999). It is believed that this turbulent flow is partially responsible for the initiation of atherosclerosis because it induces changes to the endothelial layer, which may precipitate the extravasation of the LDL and monocytes into the arterial intima (Jerome and Lewis, 1984).

As atherosclerotic lesions progress to more advanced states, other sources of lipids besides LDL become available to macrophages and there is further recruitment of monocytes. Some of the lipid particles that accumulate in the subendothelial space are aggregates of LDL (agg-LDL) (Hoff et al., 1992). Interestingly, these aggregates can interact with the endothelial layer, aiding in their retention within the sub endothelial space (Zhao et al., 2004). In addition to agg-LDL, there are both CE-containing particles that differ from native lipoproteins and FC-containing particles that have a multilaminar structure present within the atherosclerotic lesion (Chao et al., 1990). Small FC crystals associated with calcium crystals have also been identified in advanced atherosclerotic lesions (Hirsch et al., 1993). The various lipids provide several sources of extracellular lipid for the macrophages present in the lesion to engulf. Thus, there is an increase in both the number of macrophages and the amount of lipid stored within each foam cell (Jerome and Lewis, 1985; Jerome and Lewis, 1987).

When the lesion reaches an advanced state, a necrotic core can begin to form. Part of the developing necrotic core is formed from the death of some of the lipid laden foam cells. As these macrophages die the accumulated lipid is no longer maintained within the cell (Bjorkerud and Bjorkerud, 1996)(reviewed in (Littlewood and Bennett, 2003)) and results in a release of the contents into the extracellular space (Yao and Tabas, 2000; Geng et al., 2003). The released lipid becomes another source of lipid for surrounding macrophages to engulf. While the exact mechanism of cell death is not known, either of these processes will provide large quantities of acellular lipid to adjacent macrophages (Kellner-Weibel et al., 1998; Feng et al., 2003). In the case of cell death from necrosis, the various cellular enzymes would be released from the cell (Rosenfeld et

al., 1991). The release of the enzymes can generate an environment that would be conducive to lipid modification and uptake by macrophages (Rosenfeld et al., 1991). This is in contrast to death by apoptosis because the enzymes would then be packaged in apoptotic bodies. The more the necrotic core grows the more unstable the lesion becomes and the more potential there is for a clinical event to occur (Shoen and Coltran, 1999). Furthermore, the lipid produced from cell death is not packaged as a normal lipoprotein particle. The packaging of the lipid could potentially inhibit processing of the particle. The inhibition of processing could therefore quickly override the degradation and transport systems of the lysosomes when compared with single particle uptake occurring through receptor mediated endocytosis. The inhibition of processing is potentially the mechanism behind the observation that agg-LDL particles accumulate undegraded in the lysosome (Haberland et al., 2001).

Modifications of LDL

During initial lesion development, LDL modification plays a major role in lesion development. Several modifications of the LDL can occur that could lead to high cellular lipid accumulation. One of the major modifications, which occurs in the lesion, is oxidation of the LDL particle. Pathways in which oxidation can occur include, but are not limited to, attack of lipids by lipoxygenases and superoxide (Rosenfeld et al., 1991; Egan et al., 2005). Modification of the lipoproteins through the action of lipoxygenase or superoxide results in oxidation of the particles, which places negatively charged residues on the surface of the LDL particles. These negatively charged moieties allow for binding of the particles to scavenger receptors facilitating their unregulated uptake.

Oxidation of LDL is known to occur in the vessel wall and represents a physiologic modification that can be studied using tissue culture (Rosenfeld et al., 1991). However, there are large variations in both the methods and levels of oxidation of LDL particles produced *in vitro* trying to mimic that found in the lesion. These variations in both the type and level of oxidation often make comparing studies difficult (Boullier et al., 2006). A second physiological modification of LDL is aggregation, which can occur through several pathways. The best characterized pathways are through the actions of lipases on the LDL and/or the clumping of oxidized particles (Hoff and O'Neil, 1991; Boyanovsky et al., 2005). Aggregated particles can be taken into macrophages through a variety of mechanisms including scavenger receptor-mediated endocytosis and macropinocytosis. Recently, the laboratory of Kruth showed that stimulation of macrophages with M-CSF leads macrophages in culture to greatly increase cell surface activity and stimulation of lipid accumulation from unmodified LDL (Zhao et al., 2006). Thus, if aggregates are present in the lesion, even greater cellular lipid accumulation may occur.

A well characterized, but non-physiologic, modification of LDL that increases its uptake is acetylation (Basu et al., 1976; Brown et al., 1979; Kunjathoor et al., 2002), which places a highly negative charge on the lysine residues of apo B, the major apolipoprotein of the LDL (Basu et al., 1976). This modification led to the discovery of the scavenger receptor pathway (Brown et al., 1979). Uptake of acetylated-LDL (ac-LDL)by scavenger receptors results in the unregulated uptake of lipid into the macrophage. Ac-LDL has been utilized extensively in the study of cellular cholesterol metabolism and transport, but has been shown to be different from the other physiologic modifications of LDL (Brown et al., 1979; Jerome et al., 1998; Yancey and Jerome,

1998; Yancey and Jerome, 2001; Yancey et al., 2002). Acetylation of LDL leads to altered processing of the LDL compared with that of oxidized LDL (ox-LDL) (Yancey and Jerome, 2001). However, the altered processing is not due to a difference in uptake and delivery to the lysosome as both oxidized and acetylated labeled particles are delivered to the same lysosomes in a tissue culture model (Yancey et al., 2002). Additional differences between ac-LDL and physiological modifications of LDL will be discussed further in the chapter on macrophage cholesterol metabolism.

Macrophage foam cell development

In order for macrophages to become foam cells, circulating monocytes must first extravasate through the artery wall into the intima. All of the initiating mechanisms behind this extravasation are not known, but there is a cytokine gradient produced from endothelial cells and other cells in the developing lesion (Shoen and Coltran, 1999). Furthermore, disruption of the endothelial cells leads to expression of cell adhesion molecules, such as selectins and ICAM-1, on the cells surface (Cybulsky and Gimbrone, 1991; O'Brien et al., 1996). Selectin expression leads to a rolling of the monocytes along the surface of the vessel wall. Once slowed by selectin binding, the monocytes express LFA-1, the binding partner of ICAM-1, resulting in a tighter adhesion and a tethering of the monocyte to the endothelial layer. The tethering of the monocyte allows the monocyte to transmigrate between adjacent endothelial cells and enter the arterial intima (Cotran et al., 1999).

During extravasation, the monocytes differentiate into macrophages under the influence of cytokines (Shoen and Coltran, 1999). Differentiation leads to a marked

upregulation of scavenger receptors, mainly CD36 and scavenger receptor type A (SR-A) (Kunjathoor et al., 2002). Upregulation of scavenger receptors allows for the binding of modified LDL as discussed above. Once bound to the scavenger receptors, the modified LDLs are internalized and delivered to the lysosome through the endocytic pathway, (covered in more detail below). Once in the lysosome, the particles normally undergo degradation by several proteases and lipases, mainly cathepsin D, acid sphingomyelinase, and lysosomal acid lipase, which degrade apolipoprotein (apo) B, sphingomyelin, and CEs, respectively. After degradation the molecules are transported out of the lysosome for use or storage by the cells. However, de Duve and colleagues found an expansion of the lysosomal compartment in atherosclerosis (de Duve, 1974). The expansion of the lysosomes led them to suggest that atherosclerosis is a lysosomal storage disorder (de Duve, 1974). Our laboratory and others have also found an expansion of the lysosomal compartment associated with lipid accumulation (Jerome and Lewis, 1985; Jerome and Lewis, 1987). In macrophages in culture the lysosomal accumulation is accompanied by a reduction in hydrolysis of the lipoprotein CEs as foam cells develop (Yancey and Jerome, 1998; Dhaliwal and Steinbrecher, 2000; Yancey and Jerome, 2001). These data suggest there is a significant portion of foam cell development attributed to the lysosome.

Lysosomal formation and biology

Lysosomes are membrane bound organelles that degrade protein and lipid through a variety of digestive enzymes that require an acidic pH to function. Lysosomal acidity is produced and maintained by the vacuolar-type H^+-ATPase (v-ATPase). Initially lysosomes were characterized in rat liver cells based on the presence of a single

enzyme, acid phosphatase (de Duve et al., 1955), but are now known to contain over 50 acid hydrolases (Bainton, 1981). Lysosomes are also unique cellular organelles because they have no defined shape, size, or cellular location. With this fact in mind, it has been postulated that there are two different populations of lysosomes: 1. primary lysosomes, which are vesicles containing hydrolytic enzymes that are formed by budding from the Golgi apparatus and the ER (Bainton, 1981; Lodish et al., 2000); 2. secondary lysosomes, which result from the fusion of the primary lysosomes with endosomes, phagosomes or autophagosomes (Lodish et al., 2000) (Figure 1). It has also been shown that clathrin coated vesicles associate with primary lysosomes possibly for the delivery of enzymes (Friend and Farquhar, 1967; Holtzman et al., 1967). The role of lysosomes in cellular biology is multi-factorial: they eliminate damaged and unnecessary proteins (Goldberg and Dice, 1974), play a role in cell cycle and differentiation (Finley and Chau, 1991), and help facilitate antigen presentation (Castellino and Germain, 1995). Recently, genes of the atherosclerotic lesion have been characterized. Of particular interest to the studies presented in this dissertation, the genes for v-ATPases were among the genes differentially regulated between normal and atherosclerotic tissue (Forcheron et al., 2005). At a minimum, this underscores the involvement of lysosomes in atherosclerosis, but may also indicate that pH regulation is a factor in atherogenesis.

As for the lysosome's role in cholesterol regulation and atherosclerosis, it has been demonstrated, under normal cellular conditions, that LDL CEs are degraded in the lysosome. Once degraded, the FC generated from CE hydrolysis is then transported out of the lysosome for use or storage by the cell. As cellular cholesterol levels increase, there is a reduction in synthesis of the LDL receptor. At the same time, LDL receptors

are internalized and transferred to the lysosome for degradation (Goldstein and Brown, 1977) (Figure 1). Additionally, the cholesterol generated from LDL hydrolysis, in normal cells, is transferred to the plasma membrane and ER. The cholesterol in the ER results in the down regulation of 3-hydroxy-3-methylglutaryl CoA(HMG-CoA) reductase, the rate limiting enzyme in cholesterol biosynthesis (Brown et al., 1980). Interestingly, supplementation of the lysosome enzyme lysosomal acid lipase has been shown to lead to a significant reduction in atherosclerotic lesion formation (Du et al., 2004).

Cellular cholesterol

Cellular cholesterol levels

The bulk of the cellular cholesterol is found in the plasma membrane (Lange and Ramos, 1983; Lange et al., 1989; Meer, 1989; Warnock et al., 1993). Endosomes, lysosomes, and the *trans*-Golgi network contain significant amounts of cholesterol, with the levels decreasing as the vesicles move away from the plasma membrane (Colbeau et al., 1971). There are very low levels of cholesterol in the mitochondria, ER, and *cis*-Golgi. Although the ER cholesterol pool is small, the majority of the plasma membrane pool of cholesterol circulates through the ER with a half-time of approximately 40 minutes (Lange et al., 1993). This would be consistent with cholesterol efflux data suggesting there are at least 2 pools of cholesterol, a fast and slow effluxing pool (Yancey et al., 1996). The fast pool occupies the plasma membrane and the slow pool consists of internalized cholesterol (Yancey et al., 1996). Additionally, after depletion of the fast pool, the cholesterol within this pool could be replenished after approximately 40 minutes (Yancey et al., 1996).

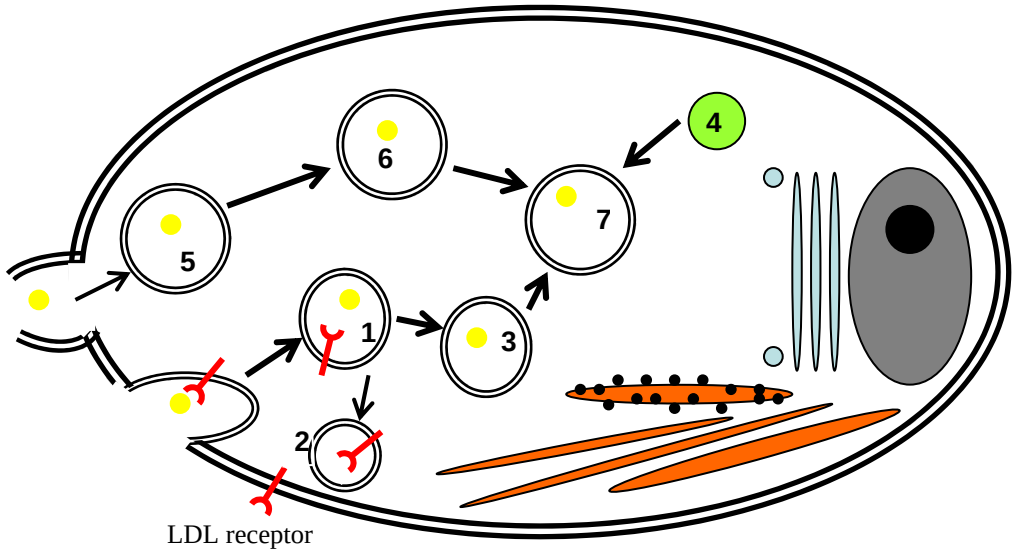

Figure Label	Compartment	Prominent Markers
1	Early Endosome	EEA-1(Simonsen et al., 1998)
2	Recycling Endosome	rab 4, cellubrevin (Gagescu et al., 2000)
3	Late Endosome	M6P R(Lodish et al., 2000), syntaxin
4	Primary lysosome	M6P R(Lodish et al., 2000)
5	Early Phagosome	VAMP 3, Rab 5 (Scott, JMB 2003)
6	Late Phagosome	Rab 7, Rab 9 (Scott JMB 2003)
7	(Secondary) Lysosome	LAMP-1, Cathepsin D(Conner, 1998)

Figure 1: Diagram of phagosomal and lysosomal formation and biologic markers. Endocytosis starts with the internalizing of a portion of membrane with a given set of receptors present and often involves a clathrin dependent process (1). After a slight acidification, causing the receptor to release its cargo, the receptor can recycle to the cell surface through a recycling endosome (2). The cargo continues down the endocytic pathway to the late endosome (3). The late endosome can then fuse with a primary lysosome, which consists of lysosomal enzymes budding from the *trans*-Golgi network (4). This leads to the formation of the secondary lysosome or what is traditionally termed the lysosome (7). Phagocytosis, which can be mediated by receptors, is a means by which the cell can internalize larger particles. This process involves the budding of the plasma membrane around the particle, the internalization of the membrane and particle, and the pinching off of the plasma membrane. This forms an early phagosome, which often has similar markers to the early endosome (5). As the phagosome matures it loses some of the markers and becomes more acidic, resulting in the formation of the late phagosome (6). These vesicles can then fuse with the primary lysosomes resulting in a phagolysosome, which cannot be distinguished from the secondary lysosome (7).

Cholesterol synthesis

FC is essential for proper cell growth and membrane function. Nearly all cells synthesize cholesterol, a complex process involving over 26 enzymes and several precursors. The rate limiting step in the synthesis of cholesterol takes place in the ER and is performed by the enzyme HMG-CoA reductase (Brown et al., 1973). Inhibition of this enzyme in the liver can significantly reduce systemic LDL levels and reduce overall levels of apo B-containing lipoproteins (Arad et al., 1990). The regulation of sterol production has been further examined and is now known to involve several steps and proteins. The major group of transcription factors involved in the regulation of sterol synthesis and uptake are the sterol regulatory element binding proteins (SREBPs), which activate over 30 sterol related genes (reviewed (Horton et al., 2002)). The regulation of the SREBPs is controlled by three additional proteins; SREBP cleavage-activating protein (SCAP), Site-1 protease (S1P), and Site-2 protease (S2P) (reviewed (Horton et al., 2002)). SCAP is located in the ER, binds to SREBP, and directs the movement of SREBP between the ER and the Golgi (Yang et al., 2002). In conditions of high cholesterol SCAP binds directly to cholesterol, through a sterol sensing domain, (Radhakrishnan et al., 2004) and is retained in the ER through binding to INSIG-1 (Yang et al., 2002). Retention of SREBP in the ER inhibits activation of SREBP and in turn cholesterol synthesis. Conversely, if cholesterol is low the SCAP- SREBP complex moves to the Golgi where SREBP undergoes cleavage via S1P and S2P, releasing the transcription factor portion of the protein, which then translocates to the nucleus (Duncan et al., 1997; Duncan et al., 1998). Three SREBPs (SREBP-1a, SREBP-1c, and SREBP-

2) have been identified. Of these, SREBP-2 regulates genes involved in sterol production and regulation (Horton et al., 1998).

Cholesterol uptake

Although cells can synthesize cholesterol, they can also acquire cholesterol through the LDL receptor pathway. The LDL receptor is expressed on the cell surface where it binds to LDL (Brown and Goldstein, 1976). It is then internalized through a clathrin dependent process (Goldstein et al., 1979) and enters the endocytic pathway, the first compartment of which is the early endosome. As the endocytic compartments start to be acidified the receptor releases the LDL particle. The receptor can then enter two separate pathways: 1. it can be recycled to the cell surface through a recycling endosome; 2. if the cell senses it has sufficient cholesterol levels it will continue down the endocytic pathway and eventually be degraded in the lysosome. This pathway is also very tightly regulated at the level of the LDL receptor (Goldstein et al., 1979). Primary human monocytes in culture show maximal LDL receptor expression within 6 days (Fogelman et al., 1981), although the cells will subsequently downregulate the LDL receptor, unless they are cultured in conditions of serum starvation (Fogelman et al., 1981). Furthermore, incubation of macrophages with LDL leads to a down regulation of the surface expression of the LDL receptor. Treatment of cells with malondialdehyde-altered LDL, a modified form of LDL, leads to an even larger down regulation of LDL receptor expression (Fogelman et al., 1981). The downregulation of the LDL receptor is in direct response to the increasing cellular cholesterol. It has also been found that up to seventy percent of LDL clearance occurs in the liver and the other thirty percent is therefore from

extrahepatic uptake (Pittman et al., 1982). Ac-LDL uptake is at least 2 fold greater than normal LDL uptake in the macrophage (Brown and Goldstein, 1983). Therefore, the role that the LDL receptor plays in atherosclerosis can be debated and scavenger receptor uptake may be more important in this system.

Macrophage cholesterol uptake

The macrophage being a scavenging cell has developed pathways to bypass the tightly regulated LDL receptor mediated uptake of LDL. These pathways, while potentially beneficial in clearance of the offending particles, do lead to the unregulated uptake of cholesterol that contributes to the development of atherosclerosis. A major pathway to bypass the LDL receptor's limited uptake of cholesterol is through scavenger receptor uptake. Two additional pathways that bypass the LDL receptor are macropinocytosis and phagocytosis. These pathways take up particles that are in the surrounding extracellular space, but receptors are not required for particle uptake.

Scavenger receptor uptake of lipid particles has been identified as a major pathway of lipid uptake leading to the production of macrophage foam cells (Krieger et al., 1993; Kunjathoor et al., 2002). There are two major scavenger receptors that have been identified to play this role, CD-36 and SR-A; however, their respective role in different systems has been debated (Kunjathoor et al., 2002). It is believed that SR-A plays a larger role in human models of foam cell development, but CD-36 may play a larger role in mouse models (Kunjathoor et al., 2002). While not clearly identified, it is also believed that there may be receptors specific for oxidized LDL. Some candidates for uptake of ox-LDL are SR-A (Krieger et al., 1993), Fc-gamma receptor II type B2

(Stanton et al., 1992), lectin-like oxidized LDL receptor-1(Yoshida et al., 1998), and CD 68 (Ramprasad et al., 1995). With regard to scavenger receptor uptake, there are several modifications of LDL which result in uptake via this pathway as discussed previously.

The uptake of unmodified LDL, small aggregates of LDL (agg-LDL), and the protein free lipid droplet go through the endocytic pathway terminating at the lysosome. It could be argued that they go through a phagocytic process that is different than receptor mediated endocytosis. However, it has been demonstrated that these particles induce macrophage foam cells that closely resemble those induced by ox-LDL (Griffin et al., 2005). This is most likely due to these pathways merging downstream at lysosomes (Minor et al., 1991; Maor et al., 1995; Yancey and Jerome, 1998; Dhaliwal and Steinbrecher, 2000; Yancey and Jerome, 2001). Therefore, the lysosomal sequestration of the lipid and the mechanisms for the lysosomal expansion may be of more importance in foam cell development than how the particles are endocytosed into the cell.

Cholesterol Storage and Efflux

As previously mentioned, FC is both essential and potentially toxic to cells. However, while cells can synthesize and endocytose cholesterol they do not have the ability to degrade cholesterol. Furthermore, only in the liver can cholesterol be excreted from the body in the form of bile acids. The bile acids can then be excreted into the feces and excreted from the body. For these reasons, cells have developed a specialized way for handling excess cholesterol, mainly cholesterol storage and efflux. A small amount of excess cellular cholesterol can be stored in the plasma membrane of the cell. However, if this level increases over that which can be accommodated in the membrane, there are

sterol sensors in the ER which activate expression and activity of aceyl-coA:cholesterol aceyltransferace (ACAT) (Chang et al., 1993). Two separate forms of this enzyme have been characterized and functionally expressed in different models (Anderson et al., 1998; Cases et al., 1998; Lee et al., 2000). These enzymes are present in the ER membrane and are responsible for esterifying a fatty acid to FC resulting in the formation of a CE. In the case of ACAT-1, the CE generated is transported and stored in a cytoplasmic lipid droplet. Increased levels of ACAT-1 are seen in monocytes that differentiate into macrophages (Miyazaki et al., 1998). ACAT-2, which is specifically expressed in the liver and intestine, generates CEs that are incorporated into apo B containing lipoproteins (Lee et al., 2000). If the cellular cholesterol level drops, cytoplasmic CE is acted on by either hormone sensitive lipase (Johnson et al., 2000) or, more likely, neutral cholesteryl ester hydrolase (NCEH) (Ghosh, 2000). The activation of these enzymes results in the hydrolysis of cytoplasmic CE to FC and a fatty acid (Figure 2).

Since cells are unable to degrade cholesterol, extrahepatic cells have developed specialized methods to rid themselves of excess FC; namely they have the ability to efflux cholesterol to extracellular acceptors. They can do this through three separate pathways. The first pathway is efflux by simple aqueous diffusion. This pathway is not an efficient means to efflux large amounts of cholesterol because of the relative insolubility of cholesterol in aqueous solutions. However, efflux by aqueous diffusion is dependent on several factors including the cholesterol concentration and the size and composition of the acceptor molecules present (Phillips et al., 1987; Lund-Katz et al., 1988). A second cholesterol efflux mechanism is through scavenger receptor type-B1 (SR-B1). SR-B1 is a bidirectional cholesterol transporter, which has the ability to efflux

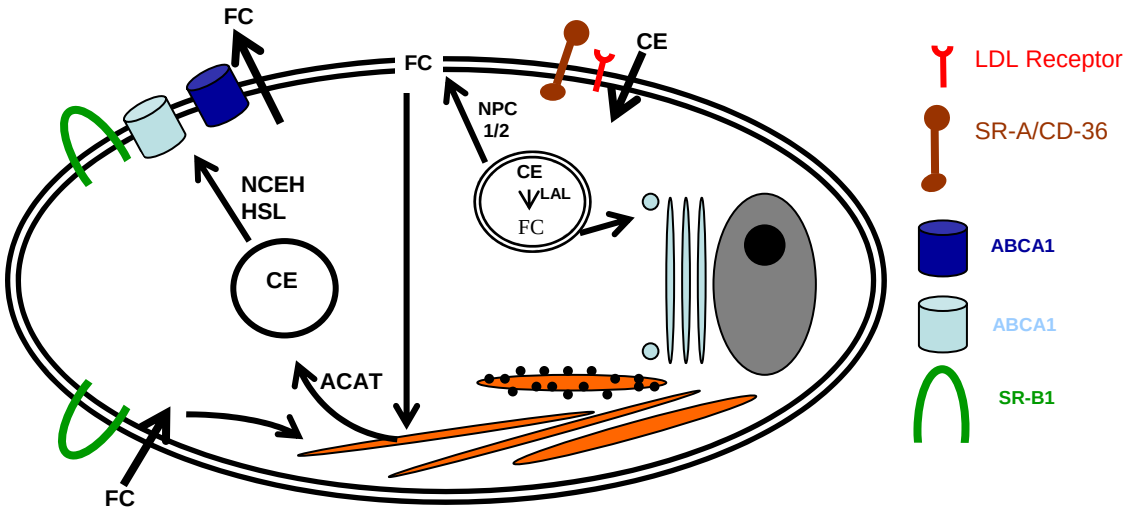

Figure 2: Diagram of cellular cholesterol movement and metabolism. Cholesterol can enter the cell on lipoproteins through the actions of the LDL receptor, scavenger receptor A (SR-A) or CD36. It can also enter as free cholesterol (FC) through scavenger receptor type B-1. It can also be synthesized by the cell in the ER (orange area). If the cholesterol enters as cholesteryl ester (CE) it must first be degraded in the lysosome to FC before it can exit from the lysosome and enter the cytoplasm. The FC can then be transported out of the lysosome through the actions of NPC-1 and 2 or potentially through non-specific vesicular transport. It is debated if the FC is transported to the plasma membrane, Golgi apparatus (light blue) or directly to the ER. However, once the cell has excess FC. The FC is transported to the ER. In the ER the FC is esterified though the actions of the esterase ACAT. Once esterified the CE is incorporated into a cytoplasmic lipid droplet for storage. If the cell needs additional cholesterol or is actively effluxing cholesterol from the cell, the CE in the cytoplasmic droplet can be hydrolyzed by neutral CE hydrolase (NCEH) or hormone sensitive lipase (HSL) or potentially other hydrolases for FC generation. If the cell is effluxing cholesterol it can do so through the actions of SR-B1, ABCA1, and/or ABCG1.

cholesterol to HDL and is known to bind LDL and anionic phospholipids (Acton et al., 1996; de la Llera-Moya et al., 2001; Liu and Krieger, 2002). SR-B1 is efficient at mobilizing cholesterol from cells that have been cholesterol enriched, but can load cells with cholesterol if the cellular cholesterol level is lower than that of the interacting particle. The third pathway for cholesterol efflux from cells is the ATP binding cassette (ABC) transporter family, mainly ABCA1 and ABCG1. These are unidirectional transporters of cholesterol, which require energy, in the form of ATP hydrolysis, to transport the cholesterol from the plasma membrane to the accepting particle (Orso et al., 2000). It is also possible that ABCG1 has a role in moving cholesterol intracellularly because only after stimulation with LXR agonists does ABCG1 localize to the plasma membrane (Wang et al., 2006). The ABC transporters interact with different molecules to facilitate reverse cholesterol transport. ABCA1 is known to interact with lipid poor apo AI, the primary lipoprotein in HDL, to transfer cholesterol and phospholipid (Remaley et al., 1997; Wang et al., 2000). Mutations in the ABCA-1 transporter result in Tangier's disease, which is characterized by very low levels of high density lipoprotein (HDL) and significant accumulation of cholesterol in peripheral tissues (Bodzioch et al., 1999; Rust et al., 1999). ABCG1 primarily interacts with nascent HDL particles and recent data suggest that ABCA1 and ABCG1 may act synergistically to increase cholesterol efflux by creating a larger cholesterol enriched particle (Wang et al., 2004). Additionally, recent data suggest that ABCG1 may be the major transporter in reverse cholesterol transport (RCT) after LXR activation (Wang et al., 2006), but there have not been any disease phenotypes associated with the loss of ABCG1.

Even with the various efflux pathways present in cells, it has been shown that cholesterol in human foam cells is more difficult to efflux than in mouse cells (Graham et al., 1996; Yancey and Jerome, 2001). However, cholesterol efflux in human cells can potentially be increased by increasing the expression of neutral cholesteryl ester hydrolase (NCEH) (Ghosh et al., 2003), which hydrolyzes cytoplasmic CEs to FC, making the FC available for efflux from the cell. Furthermore, many of these efflux systems have been analyzed in mouse cells, which have been demonstrated to be different between human and mouse macrophages (Contreras and Lasuncion, 1994). Some recent studies have revealed that genes in the atherosclerotic lesion are regulated such that the cellular cholesterol equilibrium is tilted toward cholesterol storage (Forcheron et al., 2005). For instance, there is an upregulation of perilipin, a protein associated with lipid storage, and down regulation of the efflux promoter ABCA1 (Forcheron et al., 2005). These data suggest that cells present in the lesion are more prone to accumulate cholesterol rather than effluxing it to potential extracellular acceptor particles.

Modulation of cellular function by cholesterol

Cholesterol is required for proper membrane function in cells, and often cholesterol precursors, even those closely related structurally cannot substitute in cell functions (Yeagle, 1991). Similarly, cells require cholesterol for growth and, when no exogenous cholesterol is provided, inhibition of cholesterol synthesis inhibits cell growth. However, growth can be restarted by cholesterol supplementation in the media (Soma et al., 1992). Interestingly, supplementation with mevalonic acid, a cholesterol precursor, rather than cholesterol cannot restore cell growth until high concentrations are reached (Soma et al., 1992). Moreover, there are multiple feedback mechanisms in place in

mammalian cells to assure a constant supply of cholesterol for cell growth (Brown and Goldstein, 1980). However, excess FC is cytotoxic to cells (Tabas, 2002).

Cholesterol can affect the movement and signaling of membrane proteins (Yeagle, 1991). Increasing the cholesterol levels in the membrane increases the thickness of the membrane (Bretscher and Munro, 1993), which can greatly affect the activity of transmembrane proteins (Bretscher and Munro, 1993). There is evidence that cholesterol and phospholipids form lipid rafts within the plasma membrane, a model first proposed by Simons and colleagues (Simons and Van Meer, 1988). These membrane domains have been demonstrated to play a role in the sorting of proteins within the plasma membrane (Brown and Rose, 1992). Cholesterol has also been reported to affect the movement of these proteins within the plane of the membrane. For example, the lateral diffusion of Ras, a small membrane bound GTPase, is slowed after plasma membrane cholesterol enrichment with cholesterol, but Ras diffusion is increased after overnight cholesterol depletion (Goodwin et al., 2005). An increase in the membrane cholesterol of macrophages alters F-actin organization, which can lead to altered movement and signaling of the cell (Qin et al., 2005). The change in the cytoskeleton also changes the ability of the cells to migrate potentially through a Rac mediated process (Qin et al., 2005). If the migration of the macrophages in an atherosclerotic lesion is inhibited there could be profound effects on the developing atheroma. If cholesterol is removed from endothelial cells there is a decrease in leukocyte adhesion (Broadley et al., 1991), which, if it occurs *in vivo,* would reduce atherosclerotic lesion progression.

Interestingly, the study by Goodwin *et al.* examining the effect of membrane cholesterol on Ras diffusion, also utilized methyl-β-cyclodextrin (MβCD), which has

been used extensively to alter membrane cholesterol. This study found the lateral diffusion of proteins within the membrane was also altered due to membrane interactions of the MβCD. The altered diffusion was potentially through a cholesterol independent process (Goodwin et al., 2005). In conjunction with this study it has recently been suggested that MβCD possibly plays a role in membrane organization and not just in the shuttling of cholesterol between the MβCD and the plasma membrane (Shvartsman et al., 2006). These studies show that additional controls are required in monitoring the effects of cholesterol and particles responsible for cholesterol loading on membrane properties and function. These data are very interesting because MBCD are small beta-barrel like chemicals that have been thought to only transport cholesterol, but these data suggest that cyclodextrins, in general, can also bind to the membrane and alter function.

Lysosomal storage disorders

Lysosomal storage disorders (LSD)s constitute over 50 known diseases with a combined incidence of approximately 1:7,700 live births (Meikle et al., 1999). These diseases are characterized by the loss or mutation of a lysosomal enzyme or a lysosomal associated protein. The loss results in the accumulation of substrate within the lysosomal compartment. Several LSDs involve malfunctions in lipid degradation and/or cholesterol transport. Many of these lipid-related LSDs result in neurological defects and death prior to the development of cardiovascular effects. One of the main groups of the LSDs that result in disruption in cholesterol transport are Niemann-Pick diseases. There are three classifications of these diseases. Type A and B involve mutations in the acid sphingomyelinase gene, which results in an accumulation of undegraded sphingomyelin

in the lysosome (Brady et al., 1966; Takahashi et al., 1992). This sphingomyelin accumulation in turn leads to an accumulation of FC. Niemann-Pick type C (NPC) disease is caused by a mutation in the NPC-1 protein, one of the few known cholesterol transporters in the cell (Ory, 2000). This mutation also leads to an accumulation of cholesterol in the lysosomes of these patients (Ory, 2000). In mucopolysaccharidosis III B, a different LSD, subunits of the ATP synthase and FC accumulate in the lysosomes of neurons of mouse cells (Ryazantsev et al., 2007).

There have been two studies that have found that the proton pumps may be affected in mammalian LSDs (Bach et al., 1999; Bergmann et al., 2004). Lysosomal pH is elevated in fiboblasts of mucolipidosis patients (Bach et al., 1999), an autosomal recessive storage disorder in which phospholipids and other substances accumulate undegraded in the lysosomes (Bach et al., 1999). The causes of the elevated pH are unknown. In retinal pigment epithelium cells of age-related macular degeneration patients, proton pumps are inhibited, leading to an increased lysosomal pH. Furthermore, this inhibition is associated with lipofuscin accumulation in the lysosomes (Bergmann et al., 2004).

Potential role of the vacuolar-ATPase in atherosclerosis

Proton pumps biology

Almost every biological system uses ATP as an energy source in some form. Throughout development this has led to the formation of several ATPases being present in the eukaryotic cell. Most of these ATPases are linked to the transport of ions across the membrane (Gogarten et al., 1992). In 1987 Pederson and Carafoli were the first to

give these ATPases differing designations according to their function and cellular location (Pedersen and Carafoli, 1987). Many ATPase are conserved though several species, such that the bacterial, yeast, bovine and human vacuolar-type H^+-ATPase (v-ATPase) are all similar in structure and function (Gogarten et al., 1989; Henrik et al., 1992). Similarly, v-ATPase, has been conserved evolutionarily and remains very similar to the ATP synthase, a F-ATPase, in structure (Gogarten et al., 1992). Yet evolutionarily they have developed for differing purposes, vacuolar acidification and energy generation, respectively. It is believed that all of the ATPases are linked to a common ancestral gene that was duplicated, resulting in the differences in function (Iwabe et al., 1989). V-ATPases catalyze the interconversion of the proton translocation and release energy from ATP hydrolysis (Nelson, 1992; Forgac, 1999; Nishi and Forgac, 2002). Most work regarding the v-ATPase has been performed in yeast and bacterial systems. These systems have yielded important data on their structure and function as proton pumps (Stevens and Forgac, 1997). Zhao and colleagues have shown that distribution of the human osteoclast v-ATPase is sensitive to membrane cholesterol levels (Zhao and Vaananen, 2006). Even though the osteoclast v-ATPase is not exactly identical to the lysosomal v-ATPase, in that there is a slight difference in the molecular weight of the ATP hydrolytic domain (Chatterjee et al., 1992), the data do indicate that varying cholesterol concentration can affect human v-ATPases. Additionally, the data suggest that the inhibition of the v-ATPase may occur in the transmembrane section. Moreover, it has been shown that during human monocyte differentiation there is a four-fold increase in expression of several protein subunits of the v-ATPase (Lee et al., 1997; Wang et al., 2002).

In the yeast system, mutations or deletions of one of the cytoplasmic subunits of the v-ATPase prevent the other subunits from localizing to the vacuole. The mutations, however, do not inhibit the transmembrane section of the v-ATPase from localizing to the vacuole (Kane, 1992; Klionsky et al., 1992). A deletion of a component of the transmembrane section of the v-ATPase results in the entire complex being absent from the vacuole (Kane, 1992). Proteins such as regulator of v-ATPase assembly or RAVE have been shown to aid in the assembly of the v-ATPase (Seol et al., 2001; Smardon et al., 2002). Furthermore, the v-ATPase can interact with other proteins in the endosomal pathway and these interactions can regulate the protein degradation pathway (Hurtado-Lorenzo et al., 2006). Inhibition of v-ATPase using inhibitory compounds can induce a foam cell phenotype in cultured macrophages (Yoshimori et al., 1991). Yet, little work has been done examining the possible role of the v-ATPase in the development of a macrophage foam cell, which have significant engorgement of the lysosomes.

Vacuolar-ATPase structure and inhibition

The v-ATPases are structurally composed of several proteins that make up two functional domains, the cytosolyic v_1- domain and the transmembrane v_0- domain. The v_1- domain carries out ATP hydrolysis while linking to the v_0- domain through a central stalk region (Wilkens et al., 1999; Iwata et al., 2004; Wilkens et al., 2004; Drory and Nelson, 2006). When ATP is hydrolyzed, this connection generates a proton motive force, which leads to the translocation of two protons through the v_0- domain for every ATP molecule hydrolyzed (Forgac, 1999). Yokoyama *et al.* demonstrated that there is a rotation of the transmembrane section of the ATPase during proton translocation

(Yokoyama et al., 2003) (Figure 3). The rotation of the transmembrane domain in the membrane suggests that membrane properties could play an important role in the function of this protein complex.

Inhibitors of the v-ATPase incorporate into the plasma membrane inhibiting the activity of the v_0- or proteolipid complex (Dixon et al., 2004; Pali et al., 2004a). These inhibitors often have lipid like structures. Several of the v-ATPase inhibitors have been demonstrated to interact with the transmembrane section of the v-ATPase complex (Pali et al., 2004b), suggesting a role of the membrane properties during v-ATPase inhibition. Interestingly, when the v-ATPase was inhibited in some cell types there was an expansion of the Golgi compartment (Robinson et al., 2004), similar to what has been observed in our laboratory upon induction of macrophage foam cells with mildly oxidized LDL (Jerome et al., 1998). These data suggest a connection between v-ATPase inhibition and foam cell formation. Moreover, bafilomycin A, a specific inhibitor of the v-ATPase, can inhibit fusion of the phagosome with the vesicles of the late endocytic compartment (Dermine et al., 2001). These data indicate how important the v-ATPase is to the macrophage protein degradation pathway. Bafilomycin A has also been shown to cause release of TNF-α (Bidani and Heming, 1995). TNF-α release was independent of cytosolic pH, but was directly related to the activity of the v-ATPase, suggesting a potentially important role of v-ATPase activity in inflammation (Bidani and Heming, 1995).

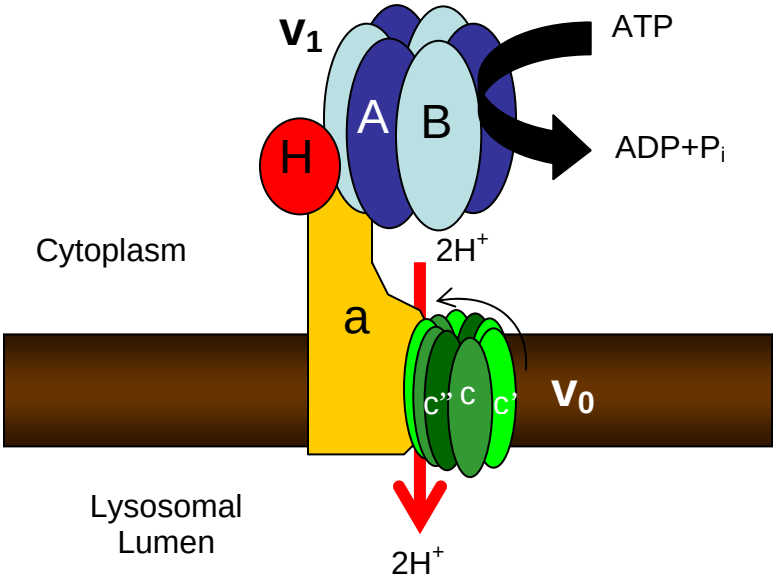

Figure 3: Diagram of the major components and functions of the v-ATPase. There are two domains of the v-ATPase. The cytoplasmic domain of the v-ATPase is termed the v_1-domain and consists of three subunits A and B each. The transmembrane domain is termed the v_0-domain and consists of between 9 and 12 subunits labeled subunits c, c', and c". The v_1-domain is responsible for ATP hydrolysis. The ATP hydrolysis generates a force through the stalk region (subunits H and a) leading to a rotation of the v_0- domain. The rotation of the v_0- domain generates a proton motive force leading to the pumping of two hydrogen ions into the lysosomal lumen. Diagram adapted from Manolson (Manolson, 2006).

Effect of lipid on the ATPases

With the variety of ATPases present in the cell and slight differences between species, studying the effect of lipids on these structures has produced controversial results (Yeagle et al., 1988; Chung et al., 2003; Crider and Xie, 2003). One unifying theme is that increased cellular cholesterol levels often have a detrimental effect on the activation of these pumps (Yeagle, 1991). Yeagle and colleagues demonstrated that the Na^+-K^+-ATPase in the kidney has little activity outside the cholesterol composition of the native membrane (Yeagle et al., 1988). Both increasing and decreasing the cholesterol concentrations significantly reduces activation (Yeagle et al., 1988). Similarly, in human erthrocytes data indicate the Ca^{2+}-ATPase has a bell shaped activation curve that correlates with cholesterol levels (Grunze et al., 1980). These studies suggest that there is a specific cholesterol concentration range in which the v-ATPase is active. Recent studies have shown that cholesterol directly inhibits the Ca^{2+}-ATPase in the sarcoplasmic reticulum in human cells through increasing the order of the membrane lipids (Li et al., 2004). In contrast to these data, it has also been demonstrated that sphingolipids, a group of phospholipids that interact with cholesterol in the membrane, are required for the formation of a functional v_1- domain of the v-ATPase (Chung et al., 2003). Since there are several trafficking steps after assembly of the v-ATPase, it would be interesting to determine if this could be a sorting effect or if the pumps were active in this environment.

In the bovine system a direct relationship has been demonstrated between lipid composition and the activity of the v-ATPase (Xie et al., 1989; Xie et al., 1993; Crider and Xie, 2003). To activate the v-ATPase maximally, a phospholipid mixture was

required; lipid vesicles composed of a single phospholipid resulted in inhibition of activation (Crider and Xie, 2003). High cholesterol concentration inhibit v-ATPase activation (Xie et al., 1993; Crider and Xie, 2003). Mattsson *et al.* demonstrated that osteoclast H^+-ATPases are very similar to v-ATPases, in both structure and function, and they are subject to inactivation by cholesterol (Mattsson et al., 1994). Additionally, inhibiting cholesterol movement in these cells with U1866A inhibits the movement of the osteoclast ATPases (Zhao and Vaananen, 2006). Finally, D'Souza and colleagues have shown that reconstitution of the lysosomal membranes is a valuable way in which to evaluate proton pump biology and that cholesterol and phospholipid are vital components in generating proper activation of the v-ATPase (D'Souza et al., 1987). Utilizing modifications of this reconstituted system with bacterial or bovine v-ATPases, data indicate that phospholipid and cholesterol content can regulate the activity of the proton pump and other transport systems (Newman and Wilson, 1980; Ambudkar and Maloney, 1986).

Knowing that cholesterol can affect v-ATPase activity, there could also be a direct link to cholesterol in the development of foam cells because cholesterol alters the actin cytoskeleton in macrophages (Qin et al., 2005). Inhibition of actin polymerization has also been linked to improper trafficking of the v-ATPase (Beaulieu et al., 2005). Therefore, these data indicate a possible link to the inhibition of v-ATPase activation and foam cell formation. Yet little has been done to directly examine how v-ATPase activity and trafficking is affected by lipid accumulation in macrophages, such as is associated with foam cell formation.

Rationale for current studies

Lysosomes have long been implicated in the development of the macrophage foam cell in atherosclerotic lesions. However, they remain an understudied aspect of atherosclerosis research. Several laboratories have demonstrated that physiologic lipid particles lead to lysosomal accumulation as foam cells develop and the cholesterol in these particles is trapped within the lysosomes. However, earlier data from this laboratory demonstrated that early in foam cell development the lysosomal hydrolysis is functional. While there is active hydrolysis of the CEs, the FC generated in the lysosomes is unable to egress from the lysosomes. This supports the hypothesis that atherosclerosis may be a lysosomal storage disorder. After this brief period of active hydrolysis, there begins to be an inhibition of hydrolysis of the CEs such that they begin to accumulate undegraded within the lysosomes. Additionally, cholesterol and phospholipids have been demonstrated to affect the activity of the v-ATPase, the major pumps responsible for generating the acidic, active lysosomal environment. To date no studies have analyzed a potential role for malfunction of these pumps in the development of macrophage foam cells. The effect of cholesterol induced inhibition of the v-ATPase could be exacerbated by these cell having as much as 1000 micrograms of cholesterol per milligram of cell protein, approximately 500 times the levels of resting macrophages. The potential mechanism of this inhibition of lysosomal hydrolysis and induction of lysosomal dysfunction is presented in this research.

CHAPTER II

Effect of Cholesterol Accumulation on Lysosomal Activation and Function

Introduction

The hallmark of an atherosclerotic lesion is the presence of macrophage foam cells, which are large lipid engorged cells. In later stages of lesion development, there is a large accumulation of lipid within the lysosomes of the foam cells (Peters et al., 1972; de Duve, 1974; Peters and de Duve, 1974). Utilizing a tissue culture model of foam cell formation, this lysosomal accumulation can be mimicked with atherogenic CE-containing particles such as mildly ox-LDL, agg-LDL and CE-enriched phospholipid dispersions (Jerome and Cash, 1995; Jerome et al., 1998; Yancey and Jerome, 1998; Griffin et al., 2005). Moreover, these particles produce a sterol accumulation (both FC and CE) similar to that seen in lesions (Jerome, 2006). More recently, it has been demonstrated that mildly ox-LDL, agg-LDL, and DISP can produce a general inhibition of lysosomal CE hydrolysis, suggesting an explanation for the lysosomal sterol accumulation (Griffin et al., 2005). One alteration that could explain the inhibition of CE hydrolysis would be an unfavorable change in the lysosome/late endosome environment, such that the pH increases beyond that at which the lysosomal lipases can function. The primary lysosomal lipase responsible for hydrolysis of lipoprotein CE is lysosomal acid lipase (LAL). This enzyme, as is suggested by the name, requires an acidic pH to function, with a peak activity between pH 3.8-4.0 and very little activity above a pH of 4.5 (Sando and Rosenbaum, 1985b). We, therefore, sought to determine if there was a change in

lysosomal pH as cellular and/or lysosomal cholesterol levels increased. To determine pH, we used the dye LysoSensor Yellow/Blue DND-160, which fluoresces yellow at acid pH but, has a significant blue shift as pH approaches neutrality (Diwu et al., 1999; Lin et al., 2001). Intracellular vesicles were classified as active if they had a pH below 4.8 and inactive if their pH was above 4.8. This pH was chosen because it is above that usually associated with lysosomes and well above the narrow pH range of human lysosomal acid lipase.

Experimental procedures

Materials

THP-1 monocytes were obtained from ATCC (Manassas, VA). Phospholipids for the production of lipid dispersions were obtained from Avanti Polar Lipids (Birmingham, AL). All other chemicals were obtained from Sigma (St. Louis, MO). Tissue culture supplies were obtained through VWR International (St. Louis, MO).

Cell culture

THP-1 monocytes were cultured and differentiated to macrophages as described previously (Yancey et al., 2002). Briefly, monocytes were plated at a density of 0.75 X 10^6 cells / ml. After differentiation into macrophages with Phorbol 12-myristate 13-acetate (50 μg / ml) for three days, cells were cleared of residual triglyceride by incubation with 4 mg / ml fatty acid free-BSA in media containing 1% FBS. Cells were loaded with modified lipoproteins, DISP (75-100 μg / ml), or magnetic beads (100 μl beads/ 30 ml media)(Invitrogen, Carlsbad, CA) in media containing 1% FBS. We tested

for loss of cells during culture by daily microscopic analysis of cell numbers and by protein levels per dish. In the time frame of the experiments, significant cell or protein loss was not observed.

Cholesterol analysis

Cholesterol loading of cells was confirmed by extracting cellular lipids into isopropanol and quantifying FC and CE content by gas-liquid chromatography (GC) as previously described (Griffin et al., 2005). Briefly, cells were rinsed twice with PBS with all PBS removed after the final wash. The cells were then dried by inverting the dishes and evaporating any residual PBS at room temperature. After drying was complete, isopropanol was added to each well. To this cholesterol methyl ether (CME) was added as an internal standard. The cellular lipid was extracted overnight in a humid chamber at room temperature. The isopropanol was then removed and divided between two tubes for analysis of free and total cholesterol. The isopropanol was dried at 37 °C under a stream of nitrogen gas. Once dried the samples for total cholesterol had 100 μl of 3:1 isopropanol: tetramethylammonium hydroxide added and vortexed to solubilize the lipid. These tubes were placed in a heat block equlibriated to 80 °C for 15 minutes to cleave the cholesterol ester bond and provide total cholesterol levels. The tubes were cooled and then both free and total cholesterol samples received 50 μl tetrachloroethylene and vortexed. Then 150 μl of deionized water was added and vortexed. The tubes were centrifuged and the bottom organic layer removed. The organic layer was dried under nitrogen gas and then resolubilized in carbon disulfide for injection on the GC. For GC separation and detection a 30 m x 0.530 mm HP-50+ capillary column was used in

conjunction with a flame ionization detector. For standardization of the column a 1:1 sample of FC:CME was used. The value of free and total cholesterol was calculated using the area of the cholesterol peak divided by the CME peak multiplied by the column standard value. These values were then normalized to the cellular protein values, which were determined using the method of Lowry *et al.* (Lowry et al., 1951). To obtain the level of esterified cholesterol the values obtained for the FC was subtracted from the value obtained for the total cholesterol.

LDL isolation and modification

LDL was isolated from plasma of healthy, fasted, normolipidemic subjects using a standard NaCl density isolation, reported previously (Yancey and St Clair, 1992). Blood was obtained using procedures approved by the human subjects Institutional Review Board. LDL was isolated at a density of $1.006 < d < 1.063$. After isolation, the LDL was extensively dialyzed against 0.9% NaCl with 0.3 mM EDTA for 72 hours (3 changes), stored under nitrogen, and maintained at 4 ^{o}C. Protein was determined by the method of Lowry *et al.* (Lowry et al., 1951). LDL acetylation was carried out using a standard acetic anhydride-sodium acetate acetylation on ice (Basu et al., 1976). To oxidize LDL, EDTA was first removed by dialysis in 0.9% NaCl for 36 hours (3 changes) at 4 ^{o}C, and then the LDL was dialyzed for an additional 1 hr at room temperature in 1 L of 0.9% NaCl. Following this, 1 ml of a 20 mM $CuSO_4$ was added (final Cu^{+2} concentration 10 μM) and allowed to equilibrate without stirring for the first hour and dialyzed for an additional 3-5 hours with stirring. Oxidation was terminated by placing the LDL in a solution of 0.9% NaCl with 1 mg / ml EDTA (pH 7.4). After 1 hr at

room temperature, the solution was placed at 4 ^{o}C for 12 hr. EDTA was equilibrated to 0.3 mM by dialysis in 0.9% NaCl and 0.3 mM EDTA for 36 hr with 3 changes. Only mildly ox-LDL was used, as determined by TBARS between 30-50 ng / mol and minimal conjugated diene formation (Bird and Draper, 1984). LDL aggregation was performed by first vortexing the LDL, under nitrogen, for 1 minute or until the LDL became cloudy. The vortexed LDL was then placed on ice and sonicated, using a probe type sonicator (Branson Digital Sonicator, Danbury, CT) at 50% power for 20 min using 10 sec pulses. The aggregates were then passed through a 0.45 μm filter to remove any remaining large or chain aggregates. The small size of the agg-LDL was confirmed by negative stain transmission electron microscopy (TEM). Particle size ranged from 40-250 nm. All modified LDL was analyzed further for uniformity by agarose gel electrophoresis and checked for degree of oxidation by TBARS and conjugated dienes as described (Bird and Draper, 1984). Agg-LDL and ac-LDL were used only if no oxidation was detected.

Lipid dispersion production and experimentation

CE-rich lipid dispersions (DISP) were produced as described previously (Minor et al., 1991) by mixing phosphatidyl choline, phosphatidyl serine, and cholesteryl oleate (1:0.1:30 by weight) in RPMI 1640 media. The resulting mixture was sonicated for 20 minutes at 50% amplitude and the size and uniformity of the dispersions determined by negative stain TEM. DISP sizes ranges from 60-200 nm. We confirmed that the DISP were not oxidized by measuring TBARS and conjugated diene formation.

Cellular staining

The locations of neutral lipid and FC accumulation in cells were determined by staining with Nile red (Greenspan et al., 1985) and filipin (Kruth, 1984), respectively. Anti-human lysosomal associated membrane protein 1 (LAMP-1) antibody (BD Biosciences; San Jose, CA) staining was used as a marker for lysosomes / late endosomes. The method for immunofluorescent LAMP-1 staining has been previously described (Griffin et al., 2005). Controls included replacing the primary antibody with pre-immune serum and leaving the primary antibody out of the protocol. LysoSensor Yellow/Blue DND-160 staining (Molecular Probes, Eugene, OR) was used to determine changes in the lysosomal pH (Diwu et al., 1999; Lin et al., 2001). Cells were washed two times in PBS and the dye was added to cells at a concentration of 5 μM in media containing 1% FBS. All images were collected within 10 minutes following the placement of dye on the cells to avoid artifacts produced by the alkaline properties of the dye. As a positive control, we stained macrophages after incubation with polystyrene beads rather than lipoprotein. This will induce a lysosomal expansion in the absence of lipid accumulation. Thus the number and size of the lysosomal fraction, compared to cellular area, should increase. For simplicity, in the following descriptions, we will use the generic term lysosome to refer to LAMP-1 positive hydrolytic compartments (lysosomes/late endosomes) since both appear to be equally affected.

Microscopy

A Zeiss Axioplan Imaging E fluorescence/brightfield microscope (Zeiss, Germany) was used for all light microscopy. Images were collected using 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 determine changes in the number of active lysosomes, a grid was placed on each image as an unbiased selector of cells to evaluate. The number of vesicles with pH below 4.8 (active lysosomes) and above 4.8 (inactive lysosomes) were counted based on intensity of the blue and green channels compared to known pH standards. This pH value was chosen because the lysosomal acid lipase 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 3 experiments were counted. The data from the different experiments were combined to obtain a mean and standard error. To test for significance, ANOVA with Tukey's posttest was performed using GraphPad Prism software (San Diego, CA). To quantify the percentage of vesicles in macrophages that were lysosomes/late endosomes, we used Nomarski differential interference microscopy to identify vesicles. A grid of points overlaid on the image was used to arbitrarily select vesicles for analysis and fluorescence microscopy was used to determine if the selected vesicles were LAMP-1 positive. One hundred to two hundred vesicles were assayed for each condition.

Western blotting

After incubation with loading vehicle for the indicated times, cells were washed three times in PBS. Cells were lysed in 2x loading buffer (0.1 M DTT, 20 % glycerol, TRIS pH 6.8, 1%SDS), loaded on a 4-12% gradient Bis-Tris SDS-PAGE gel (Invitrogen, Carlsbad, CA), run for 15 min at 100 V and 90 min at 120 V and transferred to

nitrocellulose for 90 min at 25 V. Blots were stained using a primary antibody to apo B (dilution 1:20000) (a gift from Dr. Larry Swift, Vanderbilt University), v-ATPase H-subunit (1:1000) (Santa Cruz), or β-actin (1:5000) (Sigma, St. Louis, MO). Secondary HRP conjugated antibodies were obtained from Promega (Madison, WI) and detected using ECL (PerkinElmer Life Sciences, Boston, MA) and Kodak Biomax film.

Results

The inactivation of lysosomes correlates temporally with the inhibition of CE hydrolysis and the accumulation of lysosomal CE (Yancey and Jerome, 1998; Griffin et al., 2005); suggesting generalized change in the lysosome. Lysosomal enzymes require an acidic pH to be active. Therefore, failure to maintain an active pH would inhibit the lysosomal hydrolysis of other molecules as well. To test this, we determined whether degradation of the apo B component of mildly ac-LDL, ox-LDL, or agg-LDL particles was also inhibited (Figure 4). Cells incubated with ac-LDL for up to 6 days did not accumulate immunostainable apo B. The lack of apo B staining was not due to a lack of uptake of ac-LDL since cells treated with both ac-LDL and ox-LDL had equivalent cholesterol levels. This indicates that the ac-LDL was taken up by the macrophages and delivered to lysosomes where the lipoprotein was actively degraded within the cells. This is consistent with previous studies from our laboratory and others (Basu et al., 1976; Yancey and Jerome, 1998; Yancey et al., 2002). The lack of staining was also not due to a lack of immunoreactivity of the apo B following acetylation since apo B from ac-LDL was readily detectable by our antibody prior to incubation of the ac-LDL with cells (Figure 4). In contrast to ac-LDL, macrophages incubated with mildly ox-LDL or agg-LDL

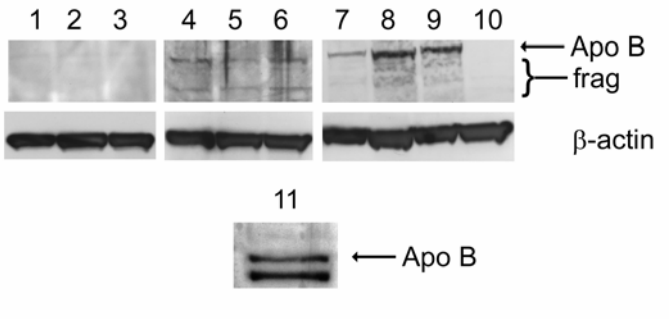

Figure 4: Apolipoprotein B accumulation in THP-1 macrophages. THP-1 macrophages incubated with mildly ox-LDL (75 μg / ml) for 1 day **(lane 4),** 3 days **(lane 5),** or 6 days **(lane 6)** or agg-LDL (75 μg / ml) for 1 day **(lane 7),** 3 days **(lane 8),** or 6 days **(lane 9)** and then blotted for the presence of apo B showed progressive accumulation of immune reactive apo B (apo B and fragments (frag)). In contrast, THP-1 macrophages incubated with ac-LDL (75 μg / ml) for 1 day **(lane 1)**, 3 day **(lane 2)**, or 6 days **(lane 3)** showed no increase in apo B staining over control **(lane 10)** levels indicating that apo B present in ac-LDL was efficiently degraded. β-actin was used as a load control. Ac-LDL (5 μg) **(lane 11)** was diluted in loading buffer and run according to the methods section to confirm detection of apo B in ac-LDL. It was immuno-blotted for the presence of apo B and two bands appeared that were identical to the unmodified LDL used in the acetylation procedure.

showed a time-dependent accumulation of immunostainable apo B and apo B fragments, with agg-LDL producing a greater intracellular accumulation than ox-LDL (Figure 4).

The lack of CE hydrolysis and apo B degradation suggest a generalized inhibition of lysosomal function. Since lysosomes require an acidic pH to function properly, the lysosomal pH was analyzed using LysoSensor Yellow/Blue. As a positive control for active lysosomes, macrophages were treated with polystyrene beads to induce a lysosomal engorgement without a subsequent increase in lysosomal lipid. The macrophages were incubated for 24 hours with polystyrene beads. They were then washed and incubated in media alone for an additional 6 days. These macrophages did not show a change in pH over time, indicating that time duration was not associated with an increase in lysosomal pH (Figure 5).

In untreated cells or polystyrene bead treated (Figure 5), the majority of vesicles in macrophages exhibited an active pH. Similarly, cells treated with mildly ox-LDL for 24 hours still contained predominantly active lysosomes (Figure 6A). However, after 7 days of mildly ox-LDL treatment (and cellular cholesterol levels increasing above 150 μg / mg cell protein) the majority of vesicles in the cells had a pH above 4.8 (Figure 6B). To determine if the lysosomal neutralization was dependent upon oxidized lipids, we investigated the effect of cholesterol loading from agg-LDL or DISP, both of which lacked oxidized lipids, on lysosomal pH. Cells loaded with small agg-LDL (Figure 6C) and DISP (Figure 6D) (both of which reach cholesterol levels in excess of 250 μg / mg cell protein) showed increases in lysosomal pH. In these macrophages the majority of vesicles had a pH above 4.8 by day 7. The lysosomal neutralization did not occur in cells treated with ac-LDL (Figure 6E), even though these cells also obtained total cellular

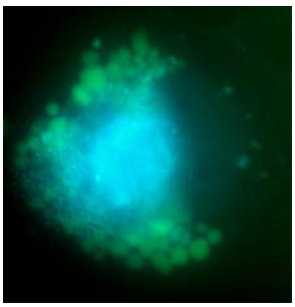

Figure 5: LysoSensor Yellow/Blue DND-160 staining of a THP-1 macrophage incubated with polystyrene beads for 24 hours and then incubated for an additional 6 days in media alone. This image demonstrates that lysosomes are long-lived and, without cholesterol accumulation, do not lose activity throughout the course of the study.

cholesterol levels above 150 μg / mg cell protein. The major difference between ac-LDL-treated cells and those treated with other lipid particles was that ac-LDL-treated cells accumulated cholesterol in cytoplasmic CE droplets and lacked lysosomal sterol (FC and CE) accumulation. This observation concurs with previous studies showing that cholesterol does not accumulate in lysosomes when ac-LDL is the loading vehicle (Brown et al., 1979; Yancey and Jerome, 1998). To verify that our methods could detect an increase in pH in the presence of ac-LDL, we incubated cells in the presence of ac-LDL and 50 μM chloroquine, a lysomotrophic agent that increases the lysosomal pH. These cells demonstrated large lysosomes, indicative of chloroquine inhibition of lysosome function, and the LysoSensor Yellow/Blue fluorescence indicated a pH above 4.8 (Figure 6F), confirming that ac-LDL does not interfere with our ability to detect increases in lysosome pH. Additionally, the macrophages incubated with polystyrene beads did not exhibit an increase over the seven day time course. These data, along with the ac-LDL data, indicate that time duration was not the cause of the pH increase.

To expand our analysis, we quantified changes in the number of active or inactive vesicles in cells with the various treatments over time. Within the initial 24 hours, lipoprotein treatment did not change the number of active lysosomes compared to controls and greater than 85% of the intracellular vesicles showed pH below 4.8 (Figure 7). After 72 hours of treatment, however, there was a blue shift in fluorescence such that, depending upon the treatment, only 45-60% of the vesicles now showed an active pH. The number of inactive lysosomes continued to increase with time and after 7 days of treatment with mildly ox-LDL, DISP, or agg-LDL the majority (>85%) of the vesicles were inactive (Figure 7). The shift in vesicle pH was also not due to loss of lysosomes as

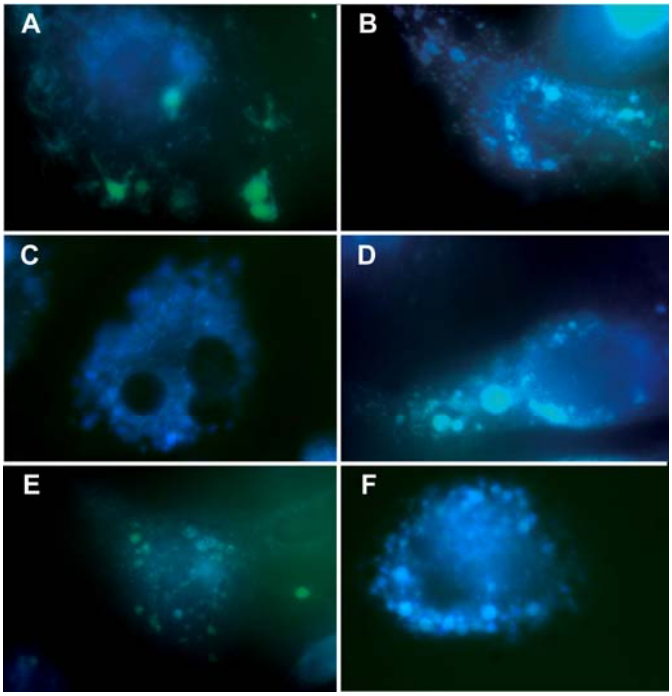

Figure 6: LysoSensor Yellow/Blue DND-160 staining of macrophages. **(A)** After 24 hours of treatment with 75 μg / ml mildly ox-LDL, lysosomes remain active (pH<4.8) as indicated by the yellow fluorescence pattern. **(B)** After 7 days of incubation with mildly ox-LDL, most lysosomes show a predominately blue fluorescence pattern indicating a rise in pH to near neutrality. **(C)** Similar lysosome pH increases are observed in THP-1 macrophages incubated for 7 days with 75 μg / ml agg-LDL. (**D**) Treatment of macrophages for 7 days with 60 μg CE / ml DISP also resulted in an increase in lysosome pH. **(E)** The increase in pH is not observed in cells treated with 75 μg / ml ac-LDL for 3 days. **(F)** To verify our ability to detect lysosomal neutralization in the presence of ac-LDL, macrophages were incubated with 75 μg / ml ac-LDL for 24 hr in the presence of 50 μM chloroquine, a lysomotrophic agent that raises the lysosomal pH. Magnification= 500X.

evidenced by positive staining for the lysosome/late endosome marker LAMP-1. For instance, in a representative experiment, 73% of vesicles in untreated cells showed positive staining for LAMP-1. This increased to 81% after 2 days of incubation with 75 μg / ml DISP and 84% at day 6. In a separate experiment, 64% of vesicles were LAMP-1 positive at day 0. This increased to 86% and 84%, respectively, with 2 or 6 day incubation with 100 μg / ml DISP. A similar pattern was seen when macrophages were incubated with 75 μg / ml agg-LDL. With agg-LDL, 66% of vesicles were LAMP-1 positive at day 0, 72% of vesicles were positive after 2 days of incubation and 90% were positive after 6 days. These data indicate that the majority of the vesicles in macrophages, even untreated macrophages, represent lysosomes/late endosomes and most of these showed increased pH in conjunction with lysosome FC and CE accumulation.

The DISP data suggests that cholesterol, phospholipids, or hydrolytic products of the two, directly or indirectly mediated the inhibition of lysosomal pH, since these are the only components in the DISP. To test whether unesterified cholesterol was a mediating factor, cells were treated with ac-LDL in the presence of progesterone. Progesterone treatment has been shown to produce FC accumulation in the lysosomes of ac-LDL-treated cells by inhibiting the trafficking of unesterified cholesterol out of the lysosomes (Butler et al., 1992; Lange, 1994; Mazzone et al., 1995). We analyzed the lysosomal pH using LysoSensor Yellow/Blue.

THP-1 macrophages were incubated with 100 μg / ml ac-LDL in the presence of 10 μg / ml progesterone for either 24 hours or 72 hours. After 24 hours incubation with ac-LDL, the cholesterol level in the macrophages ranged from 40-50 μg cholesterol per mg cell protein, which is an approximate doubling of cholesterol compared with

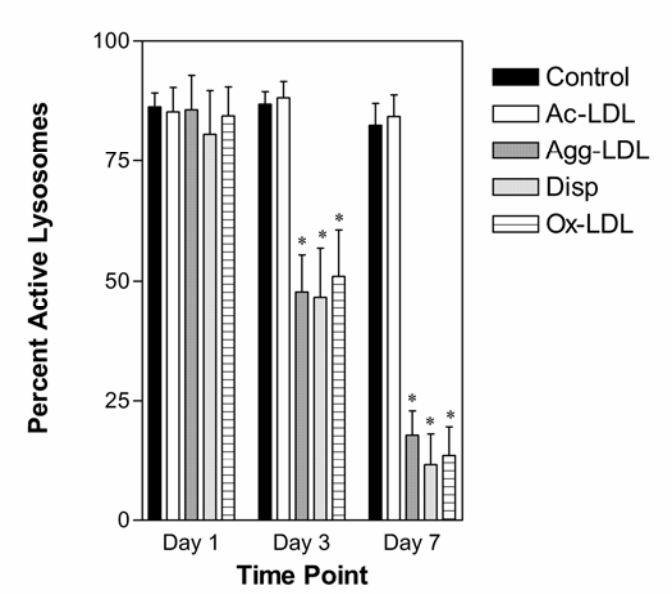

Figure 7: Quantification of the percent of vesicles showing an active pH (pH <4.8) lysosomes after treatment with various CE sources. After one day of treatment with mildly ox-LDL (75 μg / ml), agg-LDL (75 μg / ml), or lipid dispersions (60 μg CE / ml), most vesicles exhibit an active pH. However, after 3 days of loading with each type of particle there was a decrease in the number of active lysosomes. This trend continued such that less than 25% of the lysosomes exhibited an active pH after 7 days. However, Untreated (control) and macrophages treated with 75 μg / ml ac-LDL, which had similar total cholesterol levels to the ox-LDL treated cells, did not undergo a change in the number of active lysosomes over the 7 day period. Asterisks (*) indicate means which were statistically different ($p<0.001$) from those of untreated control cells.

untreated macrophages. The 72 hour incubation with ac-LDL induced cholesterol levels between 90-100 μg; a four to five times increase in total cellular cholesterol. After the 24 hour incubation with ac-LDL and progesterone there was an increase in lysosomal pH above 4.8 in almost all (>90%) lysosomes (ac+P, Figure 8A and Figure 9A). However, after removal of the progesterone block, the lysosomes of these cells rapidly regained their activity, during incubation in media alone for an additional 24 hours (ac+P+w, Figure 8A and Figure 9C). It is important to note that neither ac-LDL alone (Figure 5) nor progesterone treatment alone (P, Figure 8) resulted in a significant inactivation of lysosomes. A 24 hour wash in media alone had no significant effect on lysosomes of cells treated with progesterone alone (P+w, Figure 8). Since the inactivation of lysosomes with short-term progesterone treatment was reversible, we sought to determine if longer-term treatment with progesterone would lead to an irreversible change in lysosomes. Cells that had been incubated with ac-LDL in the presence of progesterone for 72 hours also primarily contained inactive lysosomes (>90%) (ac+P, Figure 8B and Figure 9B). To examine if the lysosomes were able to return to an active pH, the progesterone block was removed through washing and incubating the macrophages in media alone for 72 hours. After the 72 hour incubation in media alone, some of the lysosomes were able to reestablish an active pH, but the majority (>60%) retained an inactive pH (ac+P+w, Figure 8B and Figure 9D).

To confirm that progesterone treatment led to an accumulation of FC in lysosomes, we used filipin staining to identify unesterified cholesterol in cells. Ac-LDL alone did not produce FC accumulation in vesicles. However, macrophages that were loaded with ac-LDL in the presence of progesterone for 24 hours had numerous

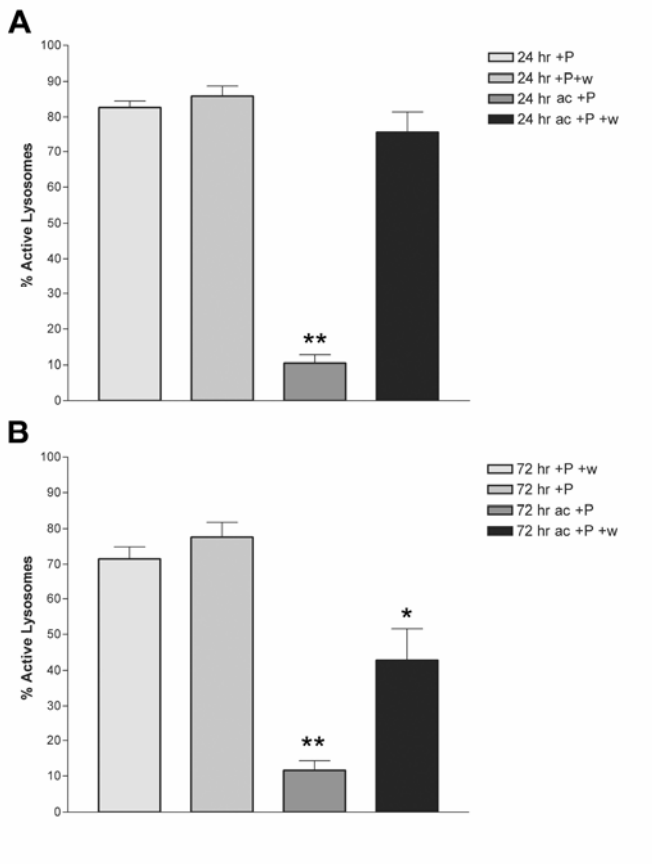

Figure 8: Effect of ac-LDL and progesterone treatment on percentage of vesicles having an active (pH < 4.8) lysosome pH. **(A)** THP-1 macrophages were treated for 24 hours in media with 10 μg / ml progesterone (+P) alone or in conjunction with 100 μg / ml ac-LDL (ac+P). Most lysosomes in cells treated with progesterone alone had an active pH (pH<4.8). The progesterone-alone cells were similar to control cells receiving no treatment and to cells treated with ac-LDL alone (Figure 2). Progesterone treatment followed by 24-hour incubation in media alone (P+w) had little effect on the number of active lysosomes. However, treatment of cells with ac-LDL (as a source of CE) in the presence of progesterone significantly (** $p<0.001$) reduced the number of active lysosomes. This effect was reversed by removing the progesterone block through incubation for 24 hours in media alone (ac+P+w). **(B)** Similar responses were seen when cells were treated for 72 hours with progesterone alone (P), progesterone plus washout (P+w), or ac-LDL in the presence of progesterone (ac+P) with a significant (** $p<0.001$) reduction in the number of active lysosomes seen only when ac-LDL provided a source of cholesterol. However, unlike the 24 hour incubations, macrophages that were incubated in media alone for an additional 72 hours after ac-LDL and progesterone treatment (ac+P+w) still had a significant reduction in the number of active lysosomes compared to control cells (* $p<0.05$).

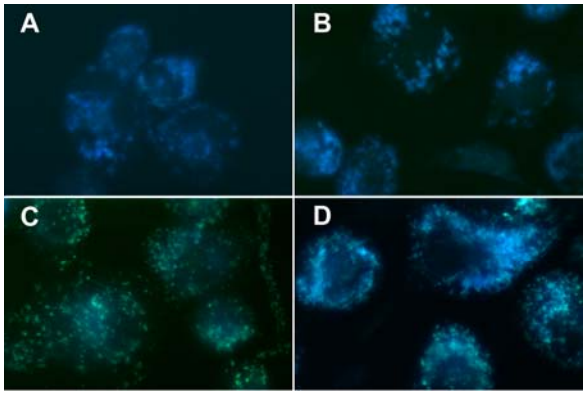

Figure 9: LysoSensor Yellow/Blue DND-160 staining of THP-1 macrophages incubated with ac-LDL in the presence of progesterone for 24 hours resulted in most lysosomes having an inactive pH **(S1A)**. Incubation with ac-LDL and progesterone for 72 hours produced a similar decrease in number of active lysosomes to that seen after 24 hours **(S1B)**. A 24 hour wash of cells incubated with ac-LDL and progesterone for 24 hours restored most lysosomes to an active pH **(S1C)**. However, even a 72 hour wash of cells incubated with ac-LDL and progesterone for 72 hours only restored some but not all lysosomes to an active pH **(S1D)**.

intracellular sites of FC accumulation (Figure 10A). These were of a size, number, and location consistent with our previous studies demonstrating FC accumulation in lysosomes (Jerome et al., 1998; Griffin et al., 2005). The filipin studies also demonstrated that there was near complete loss of intracellular FC stores after a 24 hour chase in media alone (Figure 10C). Cells that were treated for 72 hours with ac-LDL and progesterone accumulated even larger amounts of intracellular cholesterol (Figure 10B). In contrast to cells incubated for 24 hours, cells incubated with ac-LDL and progesterone for 72 hours retained much of the FC within intracellular stores even after the block was removed (Figure 10D). Untreated cells show little intracellular filipin staining, rather the staining primarily localized to the plasma membrane (data not shown). Taken together, our data suggests that lysosomal unesterified cholesterol accumulation plays a role in inhibition of lysosomal acidification.

Discussion

General discussion

Lysosomes are specialized organelles, which are responsible for degrading material, both endocytosed and intracellular, into components that can either be utilized by the cell or released into the surrounding space for use by other cells. Inhibiting this key organelle could have severe consequences to the biology of the cell. We have shown in the preceding section that as macrophages take up large amounts of physiologic CE-containing lipid particles there is an inhibition of proper lysosomal acidification. This inhibition corresponds with a decrease in lysosomal function through the accumulation of undegraded apo B and with the previously demonstrated accumulation of lipoprotein

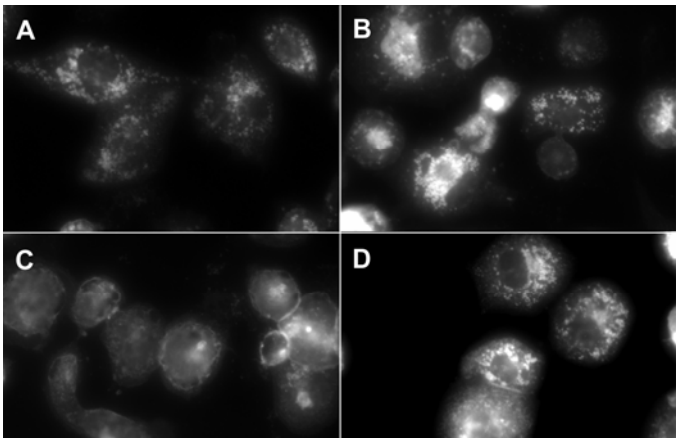

Figure 10: Filipin staining for FC in macrophages treated with progesterone and ac-LDL. Cells treated with 100 μg / ml ac-LDL and 10 μg / ml progesterone for 24 hours (**A**) and 72 hours (**B**) had many FC enriched vesicles with a size and distribution pattern consistent with macrophage lysosomes. However, removal of the progesterone block after 24 hours of ac-LDL / progesterone-treatment resulted in an almost complete reduction in intracellular FC stores after 24 hours (**C**). In contrast, intracellular accumulations of unesterified cholesterol were not completely removed when cells treated with ac-LDL and progesterone for 72 hours were allowed even a 72 hour washout period in media alone. (**D**). Magnification= 300X.

derived CE (Yancey and Jerome, 2001; Griffin et al., 2005). Additionally, lysosomal inhibition occurring in the presence of agg-LDL and DISP rules out that this is an effect of exogenously oxidized lipids and suggests that the inhibition is a more generalized component of the particle. Why these particles differ from ac-LDL is still unresolved.

The lysosomal inhibition could lead to an expansion of the lysosomal compartment because material is still being delivered to the lysosomes, but not allowed to exit. This concept corresponds with the notion that atherosclerosis has features of an acquired lysosomal storage disorder (Peters et al., 1972; Jerome, 2006). The accumulation of undegraded lysosomal contents could have dire consequences to the cell biology of the macrophage and could potentially lead to the rupture of the cell. The rupture of the macrophage foam cells could exacerbate atherosclerotic lesion formation through the formation of the necrotic core. Ac-LDL is a non-physiologic modified lipoprotein particle, which is capable of loading large amounts of cholesterol into the macrophage. While ac-LDL is non-physiologic, the fact that it can load significant amounts of cholesterol into the non-lysosomal compartments of the cell makes it a useful particle for investigating modified LDL-derived cholesterol trafficking and the effects of general cellular sterol accumulation on lysosome function. Since our agg-LDL and DISP data suggest a generalized component of the particle is responsible for lysosome inhibition, we monitor the effects of pharmacologically produced lysosomal FC accumulation on lysosomal function using progesterone treatment in the presence of ac-LDL loading. Sequestering FC in the lysosome resulted in a rapid and significant decrease in the number of active lysosomes. Conversely, in macrophages with a previous doubling of the cellular cholesterol levels, lysosomal inactivation was reversible after a

washout period and cholesterol was no longer sequestered in the lysosomes. However, cells with higher cholesterol concentrations (4-5X that of controls) were not able to reactivate lysosomes. These data suggest that there may be a threshold level of cholesterol, which, once reached, permanently blocks lysosomal function. This would have serious implications in an atherosclerotic lesion because the macrophages present are continuously exposed to lipid particles and once the threshold is reached the macrophages would be incapable of recovery. This is potentially why, in some models of atherosclerosis, pre-existing lesions were unaffected by a regression diet (Jerome and Lewis, 1990).

Lysosomal inhibition with physiological lipid particles

Lysosomal lipid accumulation is a ubiquitous but under explored feature of macrophage foam cells in atherosclerotic lesions (Jerome, 2006). It is probably most prevalent at later stages of lesion development rather than in the early, fatty streak development (Jerome and Lewis, 1987). Previously, we showed that cells treated in culture with mildly ox-LDL, agg-LDL, or CE-rich dispersions exhibit significant lysosomal free and esterified cholesterol accumulation similar to that seen in lesions (Yancey and Jerome, 1998; Griffin et al., 2005). There is ample evidence that ox-LDL, agg-LDL, and CE-containing lipid particles are present in atherosclerotic lesions. These particles are internalized by macrophages and thus deliver sterol to lysosomes (Ylä-Herttuala et al., 1989; Chao et al., 1990; Rosenfeld et al., 1991; Guyton and Klemp, 1994; Kruth, 1997). However, despite the variety of loading vehicles present in the artery wall, foam cells in advanced lesions have remarkably similar phenotypes, including substantial

free and esterified cholesterol accumulation in lysosomes (Jerome, 2006). One explanation for this would be that there is a key component common to all these atherogenic particles that produce a similar defect in the lysosomes of most foam cells. Our previous studies indicate that, in tissue culture, the CE accumulation was, in part, the result of failure of lysosomal lipoprotein CE hydrolysis (Yancey and Jerome, 2001; Griffin et al., 2005). However, both FC and CE accumulated in lysosomes and so differentiating the effects of lysosomal CE vs. FC on hydrolysis was not possible in those studies. In contrast to this, ac-LDL did not exhibit this failure of lysosomal hydrolysis (Yancey and Jerome, 2001). In the current study, using progesterone to specifically inhibit FC egression from lysosome during treatment with ac-LDL, we show that lysosomal FC accumulation alone has the potential to inhibit lysosome function by inhibiting the ability of lysosomes to maintain the correct pH. Initial accumulation of FC is an event common to all three physiological loading vehicles as well as with ac-LDL in the presence of progesterone and is thus a strong candidate for the common mediator of the subsequent lysosomal CE accumulation.

Not all vesicles in macrophages are lysosomes, although in active macrophages a significant proportion of intracellular vesicles are lysosomes (Ross and Auger, 2002). Thus, not all of the vesicles with pH above 4.8 are inactive lysosomes. Additionally, the vesicles that are observed with a pH above 4.8 would most likely be in untreated cells as well. These vesicles could account for the initial 15-20% reduction observed in active lysosomes. There is no direct way to determine the pH only of lysosomes, since it is not possible to stain cells for both lysosomal marker proteins, such as LAMP-1, and pH sensitive dyes concurrently. Furthermore, treatment of the cells with fluorescently labeled

dextrans did not completely localize with lipid filled lysosomes and could thus give an inaccurate lysosomal pH measurement (data not shown). However, in the current studies using a pH dependent fluorescent dye we have shown that a majority of macrophage vesicles were lysosomes/late endosomes and that treatment with agg-LDL or DISP increased the percentage of total vesicles in the cell that represent lysosomes. In contrast, with cholesterol loading, the percentage of vesicles with pH below 4.8 declined dramatically. In addition, we have shown previously that the majority of the FC accumulation in macrophages incubated with agg-LDL or DISP accumulates in LAMP-1-positive vesicles (Griffin et al., 2005). Taken together, these data indicate that the majority of vesicles with pH greater than 4.8 in treated cells are inactive lysosomes/late endosomes and these inactive lysosomes have significant FC accumulation.

Lysosomal free cholesterol results in inactive lysosomes

The ability of progesterone to inhibit unesterified cholesterol traffic out of lysosomes is a major and well characterized effect of progesterone treatment (Butler et al., 1992; Mazzone et al., 1995). Although the largest effect of progesterone on macrophages is inhibition of cholesterol movement (Butler et al., 1992), progesterone has other effects on macrophages, including stimulating production of growth factors and increases in synthesis of cholesterol and sterol precursors (Metherall et al., 1996; Maruo et al., 2003). However, these additional effects would also be present in cells treated with progesterone alone and, thus, cannot be the explanation for the inhibition of lysosomal acidification. Moreover, treatment of cells with ac-LDL in the presence of 2 μg / ml U1866A (3ß-[2-(diethylamino)ethoxy]androst-5-en-17-one), which also modulates

trafficking of cholesterol out of lysosomes, equally inhibits lysosomal CE hydrolysis (unpublished observation). Furthermore, treatment of cells with U1866A, which is an amine containing compound, has the possibility to affect lysosomal pH. Thus U1866A could inhibit lysosomal hydrolysis through this mechanism and the role cholesterol plays in lysosomal pH regulation would be more difficult to determine.

A decline in lysosome or cell viability is also not the explanation for the progesterone effect, since ac-LDL plus progesterone treatment did not change cell viability. Additionally, the lysosomes of cells treated for 24 hours could regain activity after the progesterone block was removed. Thus, we conclude that the lysosomal FC accumulation produced by our modified particles, or by progesterone treatment in the presence of ac-LDL, is the most likely candidate for the decreased ability of lysosome's to maintain a pH level conducive to lysosomal lipase activity.

Of particular interest is our observation that short-term cholesterol accumulation was completely reversible but that longer-term incubation, leading to higher lysosomal FC levels, was less reversible. One explanation is that those lysosomes able to re-acidify represent a younger population of lysosomes with less unesterified cholesterol. However, once a high FC level is reached, the lysosome cannot recover. Further experimentation will be required to confirm this hypothesis. If lysosomes of macrophages in the atherosclerotic lesions were equally resistant to lowering of foam cell cholesterol efflux, they would be expected to maintain their lysosomal-engorged phenotype and be resistant to regression diets. Consistent with this, in a study of pigeon atherosclerosis, it was found that the cholesterol in foam cell lysosomes was resistant to regression (Jerome and Lewis, 1990).

Our results are in contrast to a report indicating that fibroblasts from patients with Neimann-Pick Type C (NPC) disease do not have an elevated lysosomal pH (Bach et al., 1999). Mutations in the NPC-1 protein cause cholesterol to accumulate within lysosomes. It is not clear why the pH of these lysosomes remains acidic, but one possible explanation is that the FC levels in these cells never reach sufficient concentration to inhibit proton pumping. In our studies, we see no decrease in active lysosomes after 24 hours despite clear evidence that FC is accumulating in lysosomes. It appears that the lysosomes begin to fail at maintaining an active pH only after sufficient FC accumulates. It would be interesting to determine if challenging NPC-1 mutant cells with lipoprotein to further increase lysosomal cholesterol levels resulted in an increase in lysosomal pH. Similarly, it is not clear why the ac-LDL plus progesterone treatment produced a more rapid increase in lysosome pH (1 day compared to ≥ 2 days for particles) except that progesterone treatment, by severely inhibiting lysosomal loss of FC, would be expected to increase lysosomal FC very rapidly. These data suggest that there may be a threshold level of FC that must be reached prior to lysosomal inactivation. Isolating cholesterol enriched lysosomes to determine if there is a correlation directly with FC and lysosomal inhibition could yield significant data on the mechanism of lysosomal inhibition. Furthermore, determining if there could be a change in the molar ratios of cholesterol to phospholipid and the type of phospholipid present would be important because there is ample evidence in other systems that these factors play a critical role in the activation of the v-ATPase (Xie et al., 1986; D'Souza et al., 1987; Yeagle, 1991; Mattsson et al., 1994).

CHAPTER III

Lysosomal sterol accumulation does not affect the levels of the H-subunit of the vacuolar-ATPase but does inhibit the activation of the vacuolar-ATPase

Introduction

Lysosomal FC accumulation precedes lysosome inactivation suggesting FC accumulation may mediate the increase in lysosome pH. Also, inhibition of ac-LDL-derived FC egression from lysosomes is accompanied by lysosome inactivation (Cox et al., 2007). To address potential mechanisms by which FC might influence lysosome pH, we investigated the effect of FC on the lysosomal v-ATPases. V-ATPases are integral membrane pumps which utilize ATP hydrolysis to pump protons into the lysosome lumen and thus, maintain an acidic pH. One possibility for the loss of lysosomal activity could be that there is a decrease in the levels of the v-ATPase. Since the v-ATPase is a multi-protein complex, we monitored the H-subunit of the complex because it is required for the formation of an active v-ATPase complex (Lu et al., 2002; Wilkens et al., 2004). A second possibility for the loss of lysosomal activity is that there is a failure of the v-ATPase to pump protons into the lysosomal lumen. To explore this possibility, we measured quenching of acridine orange fluorescence in the presence of isolated lysosomes when the lysosomal v-ATPases were stimulated with ATP (D'Souza et al., 1987; Crider and Xie, 2003).

Experimental procedures

Lysosomal isolation and modification

THP-1 macrophages were incubated with MyOne magnetic beads (Invitrogen, Carlsbad, CA) for 72 hours at a concentration of 0.03 mg / ml. After allowing internalization of the beads for 72 hours, the cells were rinsed and media containing 1% FBS was added for an additional 72 hours. This allowed beads on the cellular surface or in the endosomal compartment to be incorporated into lysosomes. The cells were then rinsed 2 times in cold STE buffer (0.25 M sucrose, 0.01 M Tris-HCl, 1 mM EDTA, 0.1% ethanol) and scraped into 1 ml / dish of STE buffer containing protease inhibitors (Sigma, St Louis, MO). A total of six 100 mm dishes were used for each condition. The cell suspension was then placed in a cell disruption chamber (Kontes) and disrupted using 3 passes of 20 minutes each at 150 psi. This method consistently resulted in disruption of greater than 95% of cells. Western blotting for LAMP-1 indicated this method provided more consistent disruption of the cells compared to a Dounce tissue grinder.

We separated a purified population of lysosomes from the homogenate by placing the suspension in a magnetic field for 15 minutes. At the end of this period, the pellet containing bead loaded lysosomes was resuspended in buffer. To determine the effect of membrane FC levels on v-ATPase activity, the FC levels in isolated lysosome membranes were increased or decreased. To enrich lysosomal membranes with FC, isolated lysosomes were incubated with varied concentrations of methyl-β-cyclodextrins (MβCD) which were enriched with FC (Klein et al., 1995). MβCDs have been used extensively as a means of modulating membrane cholesterol levels (Yancey et al., 1996; Christian et al., 1997; Goodwin et al., 2005). After a 15 minute incubation, the lysosomes

were reisolated and washed in buffer to remove the MβCD s. Aliquots were taken for cholesterol (via GC) and protein (via Lowry) analysis to confirm the increase in lysosomal FC. As controls, lysosomes were incubated with α-cyclodextrin (CD), which does not mobilize FC and then subjected to the activation protocol. To test if the inhibition of the v-ATPase through increasing membrane FC was reversible, FC poor MβCD was utilized to return the FC enriched lysosomes back to an untreated lysosomal FC level. For most experiments, lysosomes were incubated twice, for 15 minutes each time, with the FC poor MβCD. This insured that sufficient FC was removed from the membrane to return the cholesterol level to a level similar to untreated lysosomes.

Lysosomal activation

Lysosomal activation was carried out using a modification of a procedure described previously (Crider and Xie, 2003). Proton pumping was measured as the quenching of acridine orange fluorescence when hydrogen ions are pumped into the lysosomal lumen. Briefly, isolated lysosomes were resuspended in lysosomal reconstitution buffer (0.25 M sucrose, 0.1 M HEPES, 1 mM EDTA, 150 mM KCl) to generate high K^+ levels inside the lysosome and produce a membrane potential during stimulation of the v-ATPase with ATP. Prior to activation, the lysosomes were resuspended in lysosomal reconstitution buffer containing 6.7 μM acridine orange, to allow the dye to equilibrate between the outside of the lysosome and the lysosomal lumen. After this incubation, 5-10 μg of isolated lysosomes were diluted in a cuvette containing 1.4 ml activation buffer (0.25 M sucrose, 0.1 M HEPES, 1 mM EDTA, 150 mM NaCl) containing 6.7 μM acridine orange. A steady baseline, determined as the ratio

of sample to reference fluorescence intensity, was obtained and then the v-ATPases were primed with $MgCl_2$. As soon as the baseline was reestablished (approximately 1 minute), the v-ATPases were activated by the addition of ATP (1.4 μM final concentration) and valinomycin (to promote formation of a membrane potential). V-ATPase driven pumping of hydrogen ions into the lysosome lumen is determined as a quenching of acridine orange fluorescence when excited at 495 nm and recorded at 530 nm using an SLM Aminco 8100 dual wavelength spectrophotometer. As acridine orange is protonated it aggregates, which changes its fluorescence. As controls, beads not within lysosomes were subjected to activation. As an additional control, lysosomes were activated with ATP in the absence of valinomycin.

Western blotting

After isolation and also after cholesterol modulation using the magnetic bead isolation procedure described above, an aliquot was removed and the proteins were precipitated using a trichloroacetic acid (10% of final volume), delipidated using acetone, re-solubilized using 10% SDS in water with sonication. The re-solubilized proteins were then loaded on a 4-12% gradient Bis-Tris SDS-PAGE gel (Invitrogen, Carlsbad, CA), run for 15 min at 100 V and 90 min at 120 V, and transferred to nitrocellulose for 90 min at 25 V. Blots were stained using a primary antibody v-ATPase H-subunit (1:1000) (Santa Cruz) or LAMP-1 (1:50000) (BD Bioscience, San Jose, CA). Secondary antibodies conjugated to HRP were obtained from Promega (Madison, WI) and detected using ECL (PerkinElmer Life Sciences, Boston, MA) and Kodak Biomax film.

Results

One potential mechanism for the inhibition of lysosomal activity would be changes in the levels of the v-ATPase. The v-ATPase is a multi-protein complex with a transmembrane domain, v_0, and a cytosolic domain, v_1. The H-subunit of the v_1 domain, a protein in the stalk region, is required for activation of the complex (Lu et al., 2002; Crider and Xie, 2003; Wilkens et al., 2004). Therefore, changes in the level of the H-subunit would suggest changes in the lysosomal activity. We observed no difference in the levels of the H-subunit between our controls and any of our lipid treatments (agg-LDL, ac-LDL, ac-LDL with progesterone, or ox-LDL)(Figure 11) throughout the time course of the experiment (1, 3, or 7 days). This suggests that inactivation of lysosomes is not a function of reduction in v-ATPase levels.

Another potential mechanism for the inhibition of lysosomal activity is that FC accumulation in the lysosome membrane is directly affecting the activation of the v-ATPase. Therefore, v-ATPase activity in normal and cholesterol-enriched lysosomes was assayed by monitoring acridine orange quenching. In a representative experiment, cholesterol-normal lysosomes (1.50 μg cholesterol/ mg lysosome protein) exhibited a rapid quenching of the acridine orange, showing that the v-ATPases in non-cholesterol enriched lysosomes were pumping protons into the lysosomal lumen (Figure 12). When the lysosomal membrane cholesterol was increased 46% (2.19 μg cholesterol / mg lysosome protein) by incubating the isolated lysosomes with FC-containing MβCD there was a reduction in the ability of lysosome membranes to quench acridine orange in the lysosome lumen. A 130% increase in cholesterol concentration (3.46 μg cholesterol / mg lysosome protein) over that seen in controls, almost completely in

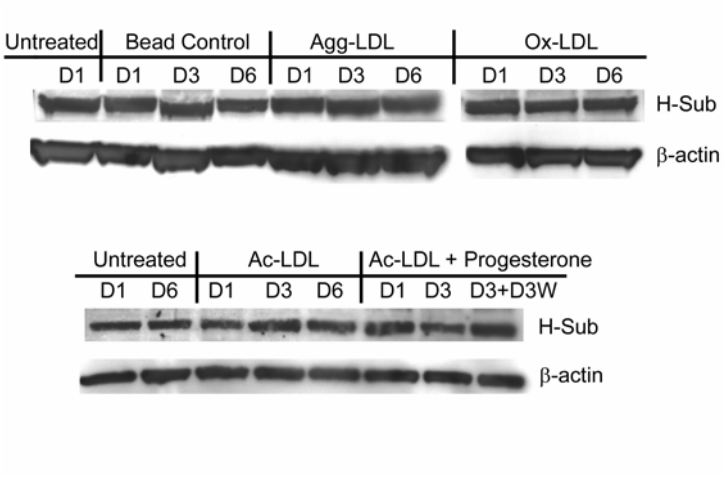

Figure 11: Western blot of the H-subunit (H-Sub) of the v-ATPase in cells loaded with agg-LDL or ox-LDL showed no difference over time in the H-subunit concentration compared with untreated cells. Likewise, ac-LDL loading or ac-LDL loading in the presence of progesterone did not alter H-subunit levels. Ten micrograms of protein was loaded in each lane. Incubation conditions were as described in methods. Cells loaded with inert beads acted as a control for lysosome stimulation. Some macrophages incubated with ac-LDL and progesterone for 3 days were allowed a further 3 day washout before analysis (D3+D3W). D1= day 1, D3= day 3, D6= day 6. β-actin was used as a load control.

inhibited the activity of the v-ATPases. Each cholesterol augmentation experiment was repeated at least 3 times with similar results each time.

Just as FC-enriched CD can transfer cholesterol to membranes, MβCD without cholesterol can remove cholesterol from membranes (Yancey et al., 1996). When we increased lysosomal membrane FC in isolated lysosomes to inhibitory levels (3.5 fold increase) using FC-containing MβCD and then reduced it by sequential incubations for 15 minutes with 1 mg / ml FC-poor MβCD to only a 1.5 fold increase (3.21 μg cholesterol / mg lysosome protein) over control levels, we regained some v-ATPase activity. The level of activity seen with this final 1.5 fold cholesterol increase over control levels was similar to that seen in lysosomes in which we obtained a modest 1.3 fold increase in lysosome membrane cholesterol (Figure 12) through brief incubation with FC-enriched MβCD.

As a control for MβCD treatment, isolated lysosomes were treated with α-cyclodextrin, which does not transfer cholesterol, but has been shown to interact with the membrane similar to MβCD (Shvartsman et al., 2006). This did not inhibit quenching of acridine orange fluorescence (Figure 13). Further controls included incubating isolated lysosomes with ATP in the absence of valinomycin, so no membrane potential would be generated, and testing whether magnetic beads alone (without v-ATPase containing membranes) in the presence of activation buffer resulted in acridine orange quenching (Figure 13). These controls did not produce a quenching of acridine orange fluorescence, confirming that inhibition of quenching was related to inhibition of v-ATPase activity by cholesterol.

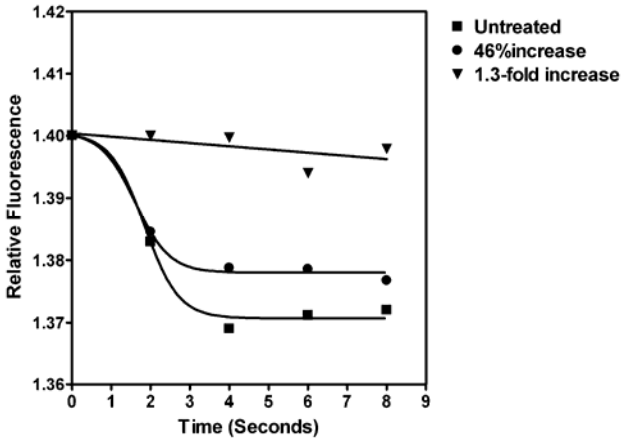

Figure 12: Quenching of acridine orange fluorescence in isolated lysosomes before or after cholesterol enrichment. Once stimulated by the addition of ATP and valinomycin (time 0), untreated lysosomes (squares) exhibit a rapid quenching of the acridine orange fluorescence, as indicated by the decrease in the relative fluorescence intensity. Acridine orange quenching is the result of activation of the v-ATPase and pumping of hydrogen ions into the lysosomal lumen. Lysosomes with a 46% increase in membrane unesterified cholesterol (circle) exhibit a reduction in overall quenching of the acridine orange. Larger increases in cholesterol levels to greater than 1.3 fold, (inverted triangle) almost complete inhibited v-ATPase activity.

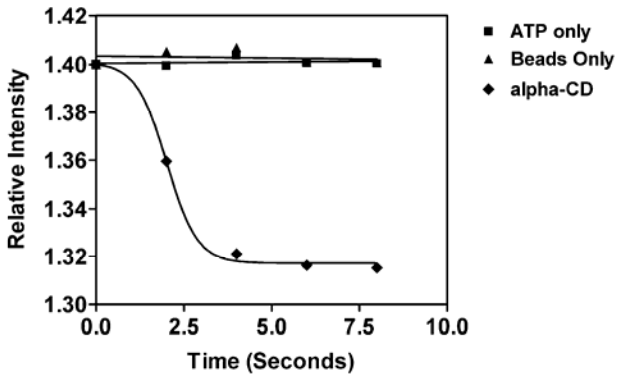

Figure 13: Isolated lysosome membranes and a membrane potential are required to quench acridine orange. Isolated lysosomes were incubated in the acridine orange activation buffer as described in the methods section and subjected to activation, except the valinomycin was omitted from the procedure (ATP only). Under these conditions, no quenching of the acridine orange was observed. To determine if the magnetic beads used for lysosome isolation have an effect on acridine orange quenching, pure magnetic beads were subjected to the complete activation protocol (Beads only) and no quenching of the acridine orange was observed. These controls demonstrate that isolated lysosomes and a membrane potential are essential for the activation of the v-ATPase and the quenching of acridine orange. To determine if cyclodextrins alone have an effect on the activation of isolated lysosomes, isolated lysosomes were incubated with α-cyclodextrin (1.0 mg / ml). Unlike β-cyclodextrins, empty α-cycodextrins do not mobilize cholesterol from membranes. After treatment, isolated lysosomes were washed, and then subjected to the complete activation protocol (α-CD). The α-CD-treated lysosomes exhibited a rapid quenching of the acridine orange fluorescence indicating activation of the v-ATPase. This control demonstrates that cyclodextrins alone do not affect activation of the v-ATPases; suggesting that it is the manipulation of lysosome membrane FC by MβCD that is responsible for the changes in lysosome v-ATPase activity observed in our studies.

Discussion

General discussion

The v-ATPase is directly responsible for the acidification of the lysosome; hence, affecting the protein levels or activities of the v-ATPase could result in improper acidification of the lysosome. We found that the inhibition of lysosomal acidification was not due to the protein levels of the v-ATPase in the cell. The activity of the v-ATPase was then examined, since it would be the other major potential mechanism for the decrease in acidification. Correspondingly, membrane properties, in particular the levels of cholesterol, have been demonstrated to relate directly to the activity of the v-ATPase (D'Souza et al., 1987; Crider and Xie, 2003). We have shown with the preceding data that modulation of the membrane cholesterol levels in isolated lysosomes alters the activity of the v-ATPase. Moreover, increasing the membrane cholesterol inhibits protons from being pumped into the lysosomal lumen. Inhibition of proton translocation is a possible mechanism for the decrease in the lysosomal activity observed in macrophage foam cells. This corresponds with other data indicating a general inhibition of lysosomal function and data suggesting that inhibiting FC egression out of the lysosome resulted in inactivation of the lysosome (Chapter II).

Increasing membrane free cholesterol inhibits v-ATPase activation

Our data demonstrate that increased lysosomal membrane FC inhibits v-ATPase activation. The mechanism is unknown, but it is possible that the FC accumulating in lysosomes partitions into the membrane and inhibits the v-ATPases. The acidic pH of lysosomes is maintained by these v-ATPases. The v-ATPases are

multimeric proteins that pump H^+-ions into the lysosome using hydrolysis of ATP as the energy source. Additionally, the efficiency of the v-ATPase can be affected in some cell types by membrane cholesterol concentration (Ambudkar and Maloney, 1986; D'Souza et al., 1987; Crider and Xie, 2003). Here we show for the first time that, in human macrophages, lysosome inactivation is not due to a decrease in levels of the v-ATPase. Furthermore, augmentation of lysosomal membrane FC concentration can inhibit lysosomal v-ATPases and produce an inactivation of lysosomes. Taken together, our observations suggest a direct effect of FC on lysosome acidification. We are currently pursuing studies to identify more details of how this inhibition occurs.

An alternate explanation for some of our data would be that increased lysosomal FC could increase leakiness of the lysosomal membrane. If protons were not retained within the lysosomes, pH would increase. However, several lines of evidence argue against this explanation. First, cholesterol increases order in membranes and makes them less permeable and has been shown to decrease lysosome leakiness (Wan et al., 2002). Moreover, increased lysosome leakiness produced by a number of different factors, including some oxidized sterols, is generally associated with apoptosis (Li et al., 2000; Yuan et al., 2000). We do not see increased apoptosis in our cells. Finally, the time course of our studies could not account for the decrease in activity because the acridine orange quenching is rapid and is the result of the protonation of the acridine orange. If the v-ATPases were actively transporting the protons into the lysosomal lumen it is unlikely that they could leak out of the lysosomes prior to protonating the acridine orange which retains the protons within the lysosome. Thus, leakiness would not appear to

explain our results. However, we cannot rule out increased leakiness as an exacerbating factor.

Taken together, the simplest explanation for our observations is that alteration in lysosomal pH accounts for the lack of CE hydrolysis we have seen in other studies and the lack of apo B degradation reported here. Although different lysosomal enzymes have different pH maxima, most require an acidic environment. The LAL responsible for CE hydrolysis has a very narrow pH range with almost no activity above pH 4.5 (Sando and Rosenbaum, 1985a). Cholesterol accumulation from mildly ox-LDL, agg-LDL, and DISP increased the lysosomal pH to a point well above that at which LAL would be active. Some lysosomal proteases have a wider pH range with some activity above pH 4.8 which might explain why some apo B is only degraded to small fragments (Conner, 1998).

CHAPTER IV

New findings and future directions

Introduction

The preceding data show that lysosomal membrane cholesterol inhibits the activity of the v-ATPase, but the mechanism for inhibition is still unknown. Furthermore, the preceding studies were conducted utilizing a lysosomal isolation with an artificial FC enrichment process and not lysosomes that have been lipid loaded intracellularly. Therefore, it still needs to be determined whether lysosomal inhibition in lipid-engorged macrophages occurs through the same mechanism as that in isolated lysosomes. For these reasons, we have initiated studies to elucidate the mechanism of v-ATPase inhibition and to determine if a similar inhibition of the v-ATPase is occurring in lysosomes in lipid engorged cells. The first section of this chapter will focus on our new studies where we analyze the mechanism of v-ATPase inhibition, the coupling of the two v-ATPase domains, and ATP hydrolysis after *in vitro* cholesterol manipulation of isolated lysosomes. We use similar techniques to analyze v-ATPase activation in lysosomes isolated from lipid-loaded cells. These data will aid in elucidating the mechanisms of lysosomal inhibition in lipid-engorged macrophage foam cells. The second portion of the chapter will focus on the future directions of these studies.

Experimental Procedures

Inorganic phosphate production

ATP hydrolysis was assayed by measuring the production of inorganic phosphate (PiBlue, San Francisco Scientific, San Francisco, CA). Standards containing known amounts of phosphate were made using serial dilution of the concentrated solution provided in the kit and ranged from 200-2000 pmol phosphate. For the experimental conditions 50 μl of activation buffer containing $MgCl_2$ was placed in the wells of a 96-well plate. To this 2-5 μg of lysosomal protein was placed in the well for each condition. To activate the lysosomes 1.1 μl of an ATP-valinomycin (ATP-v) mixture was added (final ATP concentration 0.01 μM). For termination of the reaction, 100 μl of the Pi-Blue reagent was added. Control, for the presence of inorganic phosphate in the assay, are as follows: activation buffer only, lysosomes without ATP-v, ATP-v without lysosomes, and lysosomes kept in isolation buffer (no potassium for membrane potential) with ATP-v. A minimum of 4 wells per condition were measured and averaged per experiment.

Sucrose gradient lysosomal isolation

Macrophages were either loaded with agg-LDL (100 μg / ml) or kept in 1 % FBS serum for six days, with changes after 3 days. After six days, the macrophages were scraped into 0.5 ml STE buffer containing protease inhibitors. The resulting cell suspension was subjected to nitrogen cavitation as described in the previous chapter. Once the cells were disrupted, the cell lysates were subjected to centrifugation at 500 x g for 10 minutes, to separate whole cells, nuclei, and cell debris. The supernatant was

removed. The pellet was then resuspended in 1-2 ml of STE buffer and centrifuged a second time. The resulting supernatant was added to the first. The density of the supernatant was increase to 1.15 g/ml through the addition of 0.314 g of sucrose per ml of supernatant.

For the isolation of differing density lysosomes, the supernatant was then subjected to a sucrose gradient ultracentrifugation. The densities of the sucrose layers were as follows: 1.28 g/ml, 1.15 g/ml, 1.10 g/ml, 1.01 g/ml, and 1.00 g/ml. The volume of each layer was adjusted according to the volume of the 1.15 g/ml supernatant layer, but was generally as follows: 1 ml, 5.0 ml, 3.5 ml, 3.0 ml, 0.5 ml, respectively. The centrifugation was conducted using a SW-40 rotor in a Beckman ultracentrifuge. The lysosomes were centrifuged at 19400 rpm (48000 x g) for 4 hours. The resulting lysosomal layers were isolated using an ultracentrifuge tube slicer. Aliquots of each layer were taken for GC and protein analysis. The remaining fraction was subjected to either the v-ATPase activation assay, as described previously, or to negative stain TEM. Separate experiments were conducted for Western blot analysis to verify the purity of the fractions for LAMP-1 and H-subunit analysis, as described previously.

Results

Potential uncoupling of v-ATPase domains as a result of membrane thickening

The v-ATPase is a large multimeric protein complex consisting of two domains that function together to transfer protons into the lysosomal lumen. Altered membrane properties could affect the assembly and the activity of the complex, i.e. ATP hydrolysis occurs, but protons are not pumped into the lysosomal lumen. This is feasible because it

has been suggested that the association of the two domains is reversible (Kane, 2000). Thus, if the domains were to separate, changes in the membrane might inhibit the re-association of the domains. However, we did not observe the loss of the H-subunit as we increased the cholesterol levels in isolated lysosomes, suggesting that the two subunits may remain intact (Figure 14). These data suggest that the two v-ATPase domains do not uncouple.

To definitively prove the two domains do not uncouple we measured the production of inorganic phosphate. Our experiments indicate that when the cholesterol level increased or decreased, there is a reduction in the production of inorganic phosphate (Figure 15). These data confirm that the ATPase activity of the v-ATPase complex is sensitive to perturbations in the membrane cholesterol level. The inhibition of the complex leads to inhibition of proton translocation and the lysosomes therefore become neutralized. However, the fact that increasing the cholesterol concentration of isolated lysosomes does not result in the loss of the H-subunit (Figure 14) provides evidence that cholesterol concentration does not produce a functional separation of the two domains. Additionally, even though we do see a reduction in the production of inorganic phosphate there is still some production, suggesting that hydrolysis is not completely inhibited. Therefore, it is possible that we are observing a partial uncoupling of the two domains, which might be similar to that observed using Ca^{2+} (Crider and Xie, 2003), but this potential mechanism has yet to be tested. Other membrane properties, such as leakiness, could also play a role in lysosomal inhibition (Haines, 2001; Wan et al., 2002).

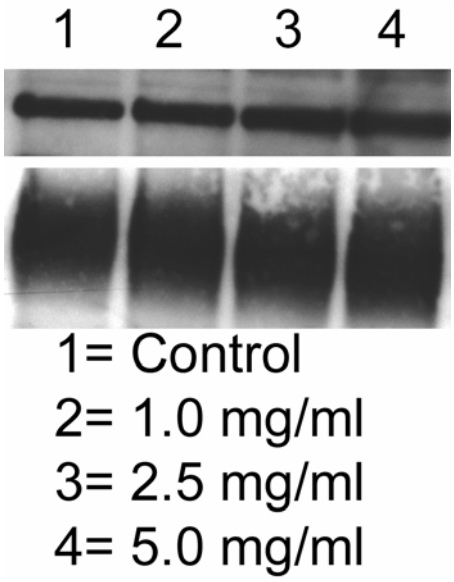

Figure 14: H-subunit protein level is not reduced upon cholesterol increase. Lysosomes were isolated using magnetic beads and then subjected to cholesterol increase using FC-enriched CD between 1.0 mg/ml and 5.0 mg/ml, which corresponded with an increase in cholesterol between 2-10 times control levels. The lysosomal proteins were then isolated and run on a 4-12% SDS-PAGE gel and blotted for either H-subunit (top band) or LAMP-1 (lower band). The blot shows that even after significant cholesterol increase Lane 4 there are similar amounts of H-Subunit present in the isolated lysosomes. These data show that a physical uncoupling of the two domains of the v-ATPase is not responsible for the reduction in v-ATPase activity.

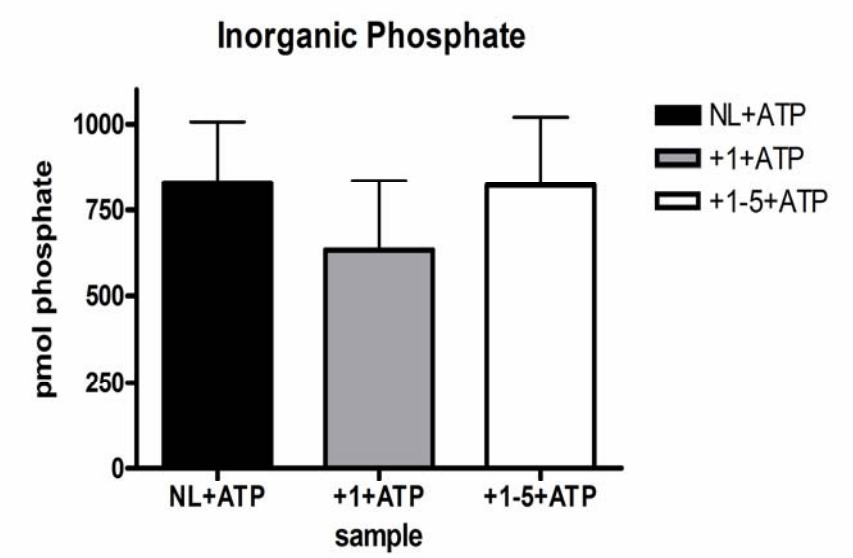

Figure 15: The production of inorganic phosphate was measured as the stimulation of ATP hydrolysis in isolated lysosomes and isolated lysosomes after membrane cholesterol modifications. Lysosomes were stimulated as done with the acridine orange quenching. In normal or untreated lysosomes there is approximately 800 pmol phosphate produced after stimulation with ATP (NL+ATP). However, after a three-fold increase (3.36 ug) in cholesterol phosphate production was reduced by approximately 200 pmol (+1+ATP). The lysosomes that had been subject to cholesterol increase were then subjected to cholesterol removal using cholesterol poor cyclodextrin and then analyzed for phosphate production. The cholesterol levels in these lysosomes were 60% above normal lysosome levels (1.77 ug) and phosphate production was returned to that of the normal lysosomes. Graph represents mean and standard deviation, means are not significantly different.

Unanswered Questions

The preceding data indicate that the two v-ATPase domains do not uncouple when membrane FC levels are increased. However, ATP is still hydrolyzed, albeit to a lesser extent than untreated lysosomes. Therefore, the exact mechanism of v-ATPase inhibition has yet to be determined. This raises two questions: 1. Does membrane organization play a role in v-ATPase inhibition? 2. Is there a second transporter involved in the activation of the v-ATPase (possibly by establishing or maintaining a membrane potential)? Cholesterol is known to have multiple effects on membrane organization and properties (Yeagle, 1991); thus, determining how membrane organization changes as cholesterol levels increase will be critical in defining the role of the membrane in v-ATPase activation. The data also indicate that a membrane potential is required for v-ATPase activation, which suggests v-ATPase activation is linked to a second ion transporter. Identifying this transporter and determining whether cholesterol has an effect on its activity would be important in defining the mechanism of v-ATPase activation.

Lysosomes from lipid loaded cells fail to activate

Our lab has been able to isolate three to four distinct populations of lysosomes in cells that have been lipid-loaded in culture. The different populations appear to reflect different degrees of sterol accumulation. These populations correspond to normal or heavy lysosomes, one-two populations of slightly lipid enriched lysosomes, and a very lipid enriched population of lysosomes. We have analyzed the heavy or normal lysosomal fractions from untreated control and agg-LDL treated macrophages for v-ATPase activity through acridine orange quenching. Notably, we find that in cells loaded

with agg-LDL, even lysosomes that are isolated at the same density as normal lysosomes in control cells, there is a failure of v-ATPase activation (Figure 16). Additionally, even though they float at a similar density to fully active lysosomes, these lysosomes have almost three times the cholesterol concentration of lysosomes from cholesterol normal cells. These data suggest that v-ATPase activity within heavily loaded foam cells is inhibited. Supporting this finding are data showing that the majority of lysosomes within lipid loaded cells exhibit an elevated lysosomal pH (Chapter II).

To characterize the isolated lysosomes more thoroughly, the various lysosome subfractions were subjected to negative stain TEM and examined for size distribution and shape. The lysosomes from the untreated macrophages were primarily composed of small unilaminar vesicles that were between 175-225 nm in diameter (Figure 17A). However, the "normal" density lysosomes isolated from the macrophages treated with agg-LDL resulted in large vesicles, between 1-2 microns in diameter, which appeared to have large quantities of lipid stored internally (Figure 17B). Our analytical data indicate that these lysosomes have three times the cholesterol levels observed in the lysosomes from the untreated cells, and thus the ATPases would be inhibited.

Unanswered Questions

The preceding indicate that even normal density lysosomes from agg-LDL treated macrophages are inhibited. Therefore, material is continually being delivered to these lysosomes (Yancey et al., 2002), but the lysosomes are incapable of degrading the material. This suggests that v-ATPase inhibition, similar to that observed using the isolated lysosomal system, is occurring in lysosomes from lipid engorged macrophages.

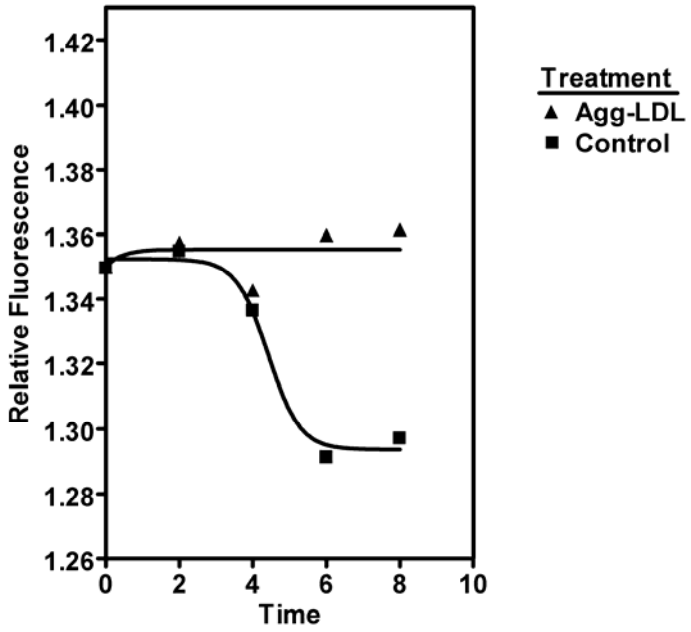

Figure 16: Isolated lysosomes from agg-LDL treated macrophages fail to activate the v-ATPase. Lysosomes were isolated using a sucrose density gradient from either control untreated macrophages or macrophages treated with agg-LDL in culture for six days. The normal or heaviest fraction of lysosomes was subjected to the activation assay using acridine orange quenching as readout. The control lysosomes had 1.11 μg cholesterol and exhibited rapid quenching of the acridine orange, similar to that observed in bead isolated lysosomes. However, the lysosomes from macrophages treated with agg-LDL had 2.88 μg cholesterol and did not quench the acridine orange.

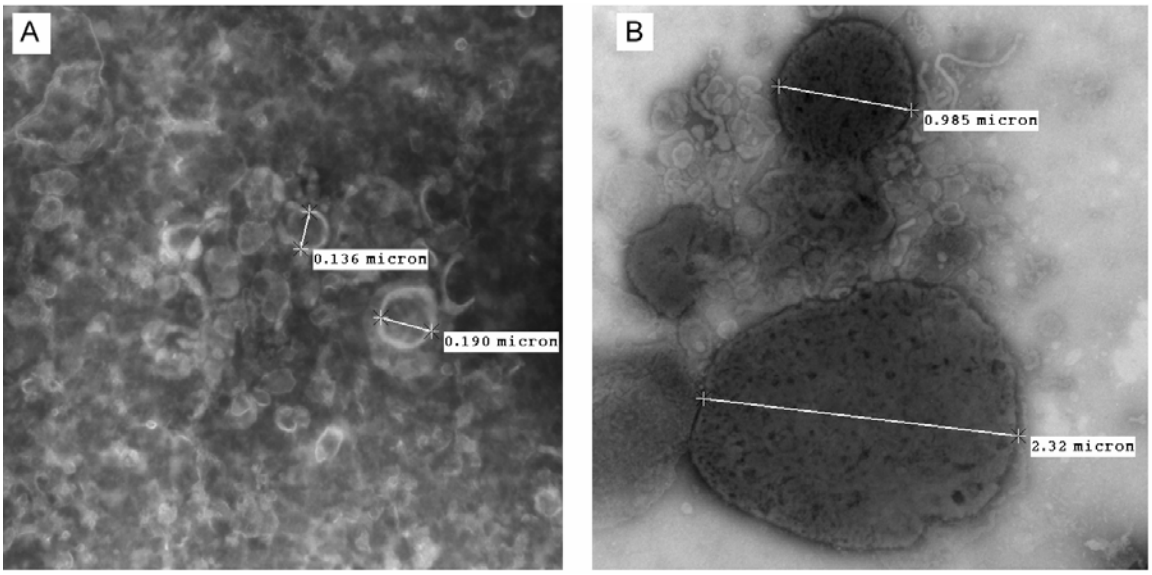

Figure 17: Lysosomes from agg-LDL treated macrophages are much larger than similar density lysosomes from untreated control macrophages and appear to have internalized lipid. Lysosomes, isolated using a sucrose density gradient, were subjected to negative stained transmission electron microscopy. Lysosomes from control cells (A) were unilaminar vesicles that were on average less than .2 microns in diameter. However, lysosomes of similar density isolated from macrophages treated with agg-LDL (B) for six days appear to have lipid in the lysosomal lumen, which corresponds to the increased cholesterol level. The lysosomes were also much larger, averaging 1-2 microns in diameter. Magnification 31000X.

However, several questions remain: 1. Is there a threshold level of cholesterol, which, once reached, inhibits the v-ATPase? 2. Does the increase in lysosomal volume affect lysosomal acidification? 3. Are newly synthesized v-ATPases trafficked properly to the lysosome during foam cell formation? As foam cells develop, the lysosomes experience a significant increase in cholesterol level and size; therefore, it will be interesting to conduct a timecourse of the lysosomal isolation and determine the activity, size and cholesterol levels and correlate this with the whole cell data. The data generated will allow determination of whether cholesterol threshold or increased lysosomal volume is responsible for the lysosome inactivation. Additionally, determining how newly synthesized v-ATPases are trafficked will be important. If there is no increase in the concentration of v-ATPases in a given lysosome, are the starting levels of v-ATPases sufficient to maintain the acidic environment of the lysosome? How these questions could be analyzed is discussed in more detail below.

Future Directions

Potential role of cholesterol induced membrane organization

Cholesterol is known to induce increased order of the phospholipid membrane as its concentration increases (Bretscher and Munro, 1993). This increase in order corresponds with an increase in the rigidity of the membrane. Both of these factors are known to induce changes in movement and function of transmembrane proteins. The group of Tabas has recently shown, using electron spin resonance (ESR), that increases in the membrane cholesterol inhibits the activity of a Ca^{2+}-ATPase in the sacroplasmic reticulum (Li et al., 2004). Using ESR, they show that the inhibition is directly related to

the increase in membrane order (Li et al., 2004). ESR studies have also shown that several inhibitors of v-ATPase also alter the membrane order (Dixon et al., 2004). Thus, it will be important to determine if cholesterol induces changes in membrane order and if these are directly associated with v-ATPase activity. While the Ca^{2+}-ATPase is different from the v-ATPase it would be interesting to utilize techniques worked out for Ca^{2+}-ATPase to examine changes in membrane order in isolated lysosomes when the membrane cholesterol content has been modified in ways that have an effect on v-ATPase activity. These studies could also be used to determine the membrane properties of lysosomes isolated from cells that have been cholesterol enriched in culture.

There are several experimental areas where examining membrane order could be utilized. Using the magnetic bead lysosomal isolation procedure, the effect of varied cholesterol concentration on membrane order could be examined. This would allow for the determination of whether the human v-ATPase also exhibits a bell shaped curve of activity, as was found for the bovine v-ATPase (Crider and Xie, 2003). Understanding how cholesterol levels affect v-ATPase activity would be important because it would demonstrate if there is a narrow concentration of membrane cholesterol in which the v-ATPase is active. Furthermore, determining the effect of cholesterol concentration on v-ATPase activity would aid in determining how stringently v-ATPase activity is linked to membrane fluidity. This would also allow us to determine how well the inhibition of activity we have reported here is linked to the membrane properties. If the v-ATPase is inhibited outside of the physiological range would be important because the sodium-potassium ATPase has been demonstrated to have little activity outside of the physiological cholesterol concentration range (Yeagle et al., 1988). Inhibition of the

sodium-potassium ATPase has been shown to have detrimental effect on the cell and kidney as a whole. It could be argued that if the v-ATPase inhibition was occurring in the lysosome of the macrophage, the cell biology could be significantly affected.

Potential role for the v-ATPase being connected to a second ion channel

There is the possibility that a membrane potential is required for complete activation of the v-ATPase; therefore, it would be interesting to search for other ion pumps that may localize to the lysosome and determine if these affect lysosomal acidification. A second ion transporter may be necessary because a membrane potential seems to be required for v-ATPase activation in systems most closely related to the human (Crider and Xie, 2003). Some have reported that while a membrane potential may be needed, an electro-chemical gradient may not be required in all systems (Ambudkar and Maloney, 1986; D'Souza et al., 1987). However, creating a gradient did enhance the acidification of the lysosomal lumen in one study (D'Souza et al., 1987). Furthermore, in the studies presented here, an electro-chemical gradient had to be initiated, using valinomycin, prior to stimulating the v-ATPase with ATP. Therefore, analyzing the proteome of different populations of lysosomes and determining which ion transporters are in or are lost in all lysosomal populations could provide a link between additional lysosomal ion transporters. These data could be linked to inactive lysosomes and could yield substantial data to the lysosomal hydrolytic and v-ATPase fields.

There are two possibilities for the inactivation of the v-ATPase if it is linked to a second transporter. The first would be that the increased lysosome surface area that accompanies the lysosomal sterol accumulation causes the additional transporter to be

trafficked improperly or to be present in insufficient quantity. The second possibility is that the second transporter is even more sensitive to cholesterol concentration than the v-ATPase. Therefore, if there are certain ionic pumps that traffic along with the v-ATPase, it would be interesting to examine how they are affected by the increase in the membrane cholesterol levels. To do this a proteomic approach could be utilized. In our lab we can consistently isolate three to four distinct populations of lysosomes depending on the lipid loading conditions. We could isolate these fractions and compare them to lysosomes isolated from untreated macrophages. The first population to analyze would be the heaviest or normal density lysosomes because these lysosomes have differential activity of the v-ATPase between agg-LDL loaded and untreated macrophages (Figure 13). Thus the v-ATPase is already affected, yet the protein to lipid ratio is similar between the fractions. To examine the possibility that there is an error in trafficking of the v-ATPase or second ion transporter, the proteome of the lysosomal populations could be analyzed. This will allow for the examination for the loss of proteins between the two fractions, which has been utilized in other systems for similar purposes (Lee et al., 2006; Sadowski et al., 2006). If a transporter is lost then it would have to be confirmed using fluorescence microscopy, since there is the possibility that a transporter could be lost during the isolation phase of the sample preparation. Additionally, fluorescence microscopy could be conducted because this technique would allow for the colocalization of other lysosomal proteins. If these experiments were conducted it would allow for great insight into foam cell formation and lysosomal function. Some potential transporters to focus on would be a chloride channel or the sodium/potassium transporters identified to be affected by cholesterol in other systems (Yeagle et al., 1988). These

transporters should be examined because both transporters have the ability to generate a membrane potential. If there is no error in trafficking, membrane properties could be related to an inhibition of the second transporter or there is an increase in the membrane permeability of the lysosome for the specific ion(s) transported. In either case, these studies would have great impact on both the atherosclerosis field and the v-ATPase field, in defining the mechanism of v-ATPase activation.

Potential role of lysosomal volume in exacerbating foam cell formation

In the data presented in this dissertation we have shown that there is an inhibition of the v-ATPase as membrane cholesterol increases and that this corresponds with an increase in lysosomal pH and decrease in lysosomal function. With this inhibition of lysosomal function, there is also an expansion of the lysosomal compartment as a percent of the cell volume. We have shown that the v-ATPase, which is responsible for the acidification of the lysosome, does not change in protein level throughout several loading conditions (Figure 10), including some which do not result in an increase in the lysosomal pH. This suggests that the expression levels may already be at a maximum once the monocytes differentiates to a macrophage. With the increase in lysosomal expansion it is possible that there may be an increase in the volume of the lysosome causing a change in the rate of acidification. This would occur if the v-ATPases present were not able to pump enough protons into the lysosome to maintain its acidity. Additionally, it is possible there is a trafficking defect of the v-ATPase as the lysosomal cholesterol increases. This in turn could have an effect on the lysosomal acidification by inhibiting an increase in the number of the v-ATPases being delivered to the lysosomes.

Very few studies have examined the effect of volume on the rate of acidification, but utilizing particle extrusion may provide novel insight into the role volume may play in lysosomal acidification (Mui et al., 2003). While we have shown that the v-ATPase activity is modulated by membrane cholesterol levels, we have done so using an isolated lysosomal system. This model is useful for examining membrane effects; however, the lysosomes should be of similar size, so any volumetric affects would be lost. It would be interesting to utilize particle extrusion to create lysosomes of defined size and utilize the acridine orange quenching method to examine the rate and level of acidification of the vesicle lumen. However, cells incubated with both polystyrene and magnetic beads, which will have an increase in overall lysosome size, exhibit active lysosomal pH and v-ATPase activity. These data argue against volume being a determining factor for lysosomal inactivation. Those studies, however, did not rule out volume as a possible exacerbating factor.

Potential role of v-ATPase trafficking in exacerbating foam cell formation

There is the possibility that trafficking of the v-ATPase may be affected during foam cell formation. Using our isolated lysosomal system in macrophages loaded with agg-LDL, we observe a decrease in the levels of the v-ATPase as the density decreases or the lipid to protein ratio increases (data not shown). However, because lysosomes are dynamic organelles, there are ill defined loading controls for this type of experiment. In that there are no set lysosomal markers that remain at a consistent concentration during the life of the organelle. Therefore, without the loading controls determining exact levels of the v-ATPase cannot be concluded. Furthermore, the antibodies currently available for

detection of the human v-ATPase are not suitable for immunohistochemistry because of nonspecific binding. With the presence of the nonspecific binding, making a definitive conclusion regarding trafficking is not possible. As antibody specificity and the advancement of various molecular biology techniques increase, it will be interesting to determine if either lysosomal volume or trafficking of the v-ATPase may play a role in the exacerbation in the development of the atherosclerotic macrophage foam cell.

CHAPTER V

CONCLUSIONS

The studies presented in this dissertation focus on the lysosomal sequestration of lipid particles, as macrophage foam cells develop. Previously, our lab found that there is a brief period during loading via mildly ox-LDL, agg-LDL, or DISP where lysosomal CE hydrolysis functions normally. The active hydrolysis of the lipid particles result in the generation of FC and fatty acids; however, with these particles the FC generated accumulates within the lysosome. In the studies presented in this dissertation it was determined that after this brief active period, the lysosomal pH increases to a level where the lysosomes are no longer active. Additionally, pharmacological accumulation of FC in the lysosome corresponds with an increase in the lysosomal pH to inactive levels. Further analysis revealed that a potential mechanism behind this increase in lysosome pH is that the FC in the lysosomal membrane is inhibiting the v-ATPase. These v-ATPases are the proton pumps of the lysosome and are responsible for maintaining the acidity of the structure.

In atherosclerosis, the hallmark of the lesion is the presence of macrophage foam cells. In these cells there is a significant increase in the internal cholesterol levels, which when sequestered in the lysosomes, could enhance the lysosome membrane cholesterol levels to more closely resemble the concentration in the plasma membrane. This could be important, because the v-ATPases are thought to be activated as they move internally from the plasma membrane (Hurtado-Lorenzo et al., 2006) possibly due to a reduction in membrane cholesterol (Liscum and Munn, 1999). Therefore, cholesterol effects on

membrane properties may play a role in this activation, which appears to be ultamately inhibited in the macrophage foam cell. Additionally, our studies show that the inactivation of the v-ATPase is reversible. The reversibility of the v-ATPase inhibition suggests that therapies which increase the egression of the cholesterol from the lysosome could possibly reactivate lysosomes and could potentially lead to the regression of the lesion. Some of these therapies could involve the increased expression of NPC-1 and NPC-2 proteins. These proteins are two of the known sterol transporters in the cell and their activation could transport cholesterol out of the lysosomal membrane, thus reducing the membrane cholesterol concentration.

Overall, the data presented in this dissertation are consistent with the hypothesis that cholesterol accumulation in lysosomes, particularly FC, contributes to the failure of lysosomes to maintain an active pH. The experiments presented in this dissertation also highlight the fact that cholesterol can modulate lysosome function through changing the activity of the v-ATPase. Furthermore, accumulation of cholesterol within lysosomes can produce an acquired lysosomal insufficiency. Importantly, we show that several different types of particles, similar to those implicated in atherosclerosis, can bring about this insufficiency. Thus, these data suggest a partial explanation for why the foam cells in advanced lesions have a similar lysosomal phenotype despite the presence of multiple sources of cholesterol. Lastly, we show here for the first time that the cholesterol delivered to lysosomes as a component of modified lipoproteins has the potential to inhibit v-ATPase activity in human macrophages. The long-term nature of the inhibition would suggest that once a foam cell's lysosomes are inhibited they do not regain function. Since a macrophage can exist within a lesion for extended periods, the failure of

acidification and subsequent trapping of cholesterol within this key organelle for macrophage function would suggest this defect can influence the nature and progression of the atherosclerotic lesion.

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