Triglyceride Regulation of Lysosomal Cholesterol in THP-1 Macrophages

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The central question I investigated in my research this semester was how the cholesterol and triglyceride metabolic pathways interact in macrophages when cholesterol levels are high. What factors prevent or enhance macrophage ability to regulate cholesterol metabolism? We have preliminary evidence that excessive cholesterol accumulation in macrophages alters triglyceride metabolism in these cells. When cellular cholesterol levels are normal, the primary site of triglyceride hydrolysis is on the surface of the macrophage. There, lipases hydrolyze triglycerides within triglyceride-rich lipoproteins to glycerol and fatty acids. The fatty acids are carried across the cell membrane into the cytoplasm. In the cytoplasm, the fatty acids can be utilized to make triglycerides, phospholipids and cholesteryl esters. Cholesterol influences how the cell breaks down triglycerides. If cholesterol levels are high then triglyceride metabolism tends to occur more frequently in the lysosome. In contrast, when the cells have excess cholesterol, particularly within their lysosomes, the surface hydrolysis is suppressed and lipoproteins are taken up by endocytosis and delivered to lysosomes for hydrolysis. The effect of inhibiting the surface lipolysis was evaluated to determine the impact on intracellular cholesterol and triglyceride levels. The data indicates that the presence of triglyceride restores lysosomal metabolism of cholesterol.

INTRODUCTION

Cardiovascular disease is the number one cause of death not only in the United States, but worldwide. Atherosclerosis, the thickening of the artery walls caused by excessive accumulation of cholesterol and triglycerides, is the leading cause of heart disease. The thickened artery wall contains many constituents, including extracellular lipid and macrophages that have accumulated large amounts of lipid, primarily cholesterol. The lipid-filled areas of the atherosclerotic lesion are weaker, increasing the susceptibility of lesions to rupture. Rupture of the wall leads to clots which stop the flow of blood, causing heart attacks or strokes and eventual cardiac arrest.

A common goal in the investigation of atherosclerosis is a better understanding of how cholesterol buildup can be prevented or removed. Prior investigations have indicated that inordinate cholesterol accumulation in macrophages changes triglyceride metabolism in these cells (Jerome 2003). Under normal cellular triglyceride metabolism, the primary site contributing to triglyceride hydrolysis is the surface of the macrophage, where lipases hydrolyze triglycerides to glycerol and fatty acids (Jerome, 2003). The fatty acids formed from surface lipase hydrolysis are taken on by the cells into the cytoplasm. These fatty acids are frequently activated and used to form new triglycerides and cholesterol esters, which exist in the cytoplasm as lipid droplets. An alternative to surface lipase enzyme degradation is the receptor-mediated endocytosis of particles into the endosomes and transfer to the lysosomes (Jerome, 2003). Lipases in the lysosome hydrolyze triglycerides into fatty acids, which can then be removed from the lysosome and utilized for the formation of cytoplasm lipid droplets and other molecules (Ouimet et al, 2011). When cells have excess cholesterol in the lysosomes, surface hydrolysis is suppressed and lipoproteins are taken up by endocytosis and delivered to lysosomes for hydrolysis (Jerome, 2010). Alternatively, under regular cholesterol metabolism, cholesteryl ester lipoproteins are internalized into cells via receptor-mediated endocytosis and transported to the lysosome to be broken down. Lysosomal acid lipase hydrolyzes the cholesterol esters into fatty acids and free cholesterol. Vesicles begin to form on the membrane of the lysosome and the free cholesterol is transported to the plasma membrane (Ouimet et al, 2011). At the plasma membrane, free cholesterol plays an essential role in facilitating ordinary function. If the free cholesterol is in excess, it forms lipid droplets in the cytoplasm. Free cholesterol is esterified in the endoplasmic reticulum by acyl cholesterol transferase. The reverse process of converting the cholesteryl esters of the lipid droplets into free cholesterol for the plasma membrane can occur from the neutral cholesteryl ester hydrolyase enzyme (Jerome, 2010).

Previous studies have focused on an effect of cholesterol accumulation on triglyceride metabolism.
This research uses a combined biochemical approach to study triglyceride accumulation and its subsequent effect on cholesterol metabolism. We analyzed changes using fluorescent microscopy to observe lipid droplet buildup and the result of surface hydrolysis inhibition. Additionally, lysosomal lipoprotein accumulation was fluorescently labeled to provide evidence of cellular alternatives to surface lipase hydrolysis and absorption of triglycerides. The resulting analysis indicated a drastic increase in lysosomal lipoprotein accumulation after the inhibition of surface lipolysis. Furthermore, orlistat treatment groups indicated an increase in cholesterol accumulation, providing evidence that triglyceride accumulation restores cholesterol metabolism.

**Materials and Methods**

Two forms of experiments were used to evaluate the presence of cholesterol and triglyceride under the same treatment groups. There were a total of eight treatment groups, each of which was prepared in cell culture according to the necessary conditions to prepare them for either gas chromatography or light microscopy. The preparation of lipoproteins for the treatment groups allowed the cell culture to be established.

**Isolation of Lipoproteins**

The lipoproteins used in the experiment were isolated after drawing 150mL of blood from each of six volunteers in the labs. The collected blood was mixed with Ethylenediaminetetraacetic acid at 1.5mg/mL to prevent clotting. The blood was centrifuged for twenty minutes at 2500 RPM in order to draw off the plasma from the red blood cells. The plasma was split into 50mL conical tubes with 500uL of protease inhibitor. All samples were kept on ice throughout the isolation process. Very low density lipoprotein (VLDL), the triglyceride used in the experiment, was isolated from the plasma samples using centrifugation. In order to maintain an accurate volume, the isolated plasma samples were added to polyallomer centrifuge tubes leaving approximately 5mL for 0.9% NaCl, and 1mg/mL EDTA stock. All bubbles in the tubes were carefully removed due to the risk of breaking the tubes during ultracentrifugation. The samples were ultracentrifuged for twenty hours at 48000 RPM and 4°C. Once the ultracentrifugation was complete, a tube slicer was set up in order to separate the yellow-orange upper VLDL layer from the remaining plasma at the bottom of the tube. After the VLDL was separated from the plasma, the low density lipoprotein (LDL) was ready to be isolated from the remaining plasma. The plasma was removed from the polyallomer tubes and added to plastic centrifuge tubes. Once the volume was calculated in the tube, NaCl was added to the plasma to increase the density of the plasma to 1.063 mg/mL. The samples were then placed in an ultracentrifuge and spun for 22 hours at 48000 RPM and 4°C. The same tube slicer was repeated as above to remove the LDL from the top layer of the plasma sample. Dialysis solutions were then prepared to dialyze LDL for three days in 0.9% NaCl, 0.3mM EDTA solution. All isolated lipoprotein were stored at 4°C under nitrogen in 50mL conical tubes.

**Cell Culture**

In order to plate and maintain cell culture for the experiments, three types of THP-1 Culture medium were prepared. The essential difference between the three mediums was the concentration of TPA (0%, 1%, 10%), which was used to plate the cells. The following solutions were added to RPMI 1640 to formulate the medium: 50mL of FBS, 12.5mL of 0.4M HEPES, 5mL of L-glut (29.2 mg/mL in 0.85% NaCl), 5 mL 10,000 ug/mL of Pen-Strep, 5 mL of 100X MEM vitamins, and 4.4 uL of beta-mercaptoethanol. In order to keep concentration integrity, the appropriate amount of RPMI 1640 was removed from the media. Five 6-well plates were plated with 2mL of cells at a cell density of 600,000 per mL in 10% plating media. The cells were incubated for four days. On the fourth day the media was changed and the treatment groups were established:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>6D Control</td>
<td>6D Control followed by 3D orlistat</td>
</tr>
<tr>
<td>6D Control followed by 3D orlistat</td>
<td>6D Control followed by 3D VLDL</td>
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<tr>
<td>6D Control followed by 3D AggLDL</td>
<td>6D Control followed by 3D AggLDL and orlistat</td>
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<tr>
<td>6D Control followed by 3D VLDL</td>
<td>6D Control followed by 3D AggLDL and orlistat</td>
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<tr>
<td>6D Control followed by 3D VLDL and orlistat</td>
<td>6D Control followed by 3D AggLDL and orlistat</td>
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Concentrations are as follows:

- AggLDL 30mg/mL
- VLDL 20mg/mL
- Orlistat 4uM

The only difference in the treatment groups between the
repeat experiments was that the wells were pretreated with orlistat for three hours prior to the media change.

**Isopropanol Extraction**

To extract lipids from the cells for analysis, the cells were incubated overnight in 1mL isopropanol in a tightly sealed container with wet paper towels on the bottom in order to prevent evaporation. Reproducibility was key to ensure accurate results, so an Eppendorf repeat pipette was used to add 1mL of isopropanol to each well. Additionally, 100uL of (10mg/100mL) cholesterol methyl ether (CME) were added to each well. The isopropanol was extracted for no more than eighteen hours to prevent plastic being extracted from the plate.

**GC Preparation**

To analyze the levels of free and esterified cholesterol in cells, the isopropanol extract was divided into three tubes. First, 100ul were removed from the well and kept in a gas tubes at 4°C for triglyceride analysis. Then, the remaining volume was divided in two and placed in glass centrifuge tubes for analysis of sterol. One tube was for analysis of total sterol and the other for free (unesterified) cholesterol. After the extracts were removed from the wells, the plates were inverted, dried for an hour, and placed in the freezer to complete a NaOH extraction of protein at a later date. The isopropanol extracts for cholesterol (total and free) were dried under nitrogen using a N-EVAP at 80-85°C. Each glass centrifuge tube was labeled for either free or total cholesterol. 100uL of 3:1 isopropanol:TMH was added to all the dried total cholesterol samples. Each total sample was then vortexed, capped, and placed on a stabilized heat block at 80-85°C for fifteen minutes for saponification of any cholesteryl ester to free cholesterol. After fifteen minutes, the samples were allowed to cool. 50uL of factory TCE (trichloroethylene) and 200uL of dH₂O were added to each tube including the free cholesterol tubes. The samples were spun in a centrifuge for twenty minutes at 2000RPM at 4°C. After removing the samples from the centrifuge, 40uL of the bottom layer (containing the sterol) were removed using a Hamilton syringe and placed in a GC vial. The Hamilton syringe was rinsed three times between each sample and six times between each triplicate with hexane. Once the samples were placed in the GC vials, they were placed on the N-EVAP to be dried down along with two column standards. After all samples were dried down, they were resuspended in 75uL of carbon disulfide for the samples and 100uL for the column standards. All vials were then placed on an Agilent Gas Liquid Chromatographer where the cholesterol levels were calculated.

**Triglyceride Microplate Assay**

In order to analyze the sample’s triglyceride values, a microplate assay was set up for analysis of the isopropanol extracts reserved for triglyceride analysis. Seven triglyceride standards were made up in concentrations from 0 to 100 μg/mL. After the standards were made up, triglyceride (GPO) reagent was prepared by adding 20mL of tissue culture grade water to the bottle. 100uL of the standards were pipetted in a 96-well plate in triplicates. 20uL of the triglyceride sample were also added in triplicates to each well followed by 80uL of tissue culture grade water. 100uL of the GPO reagent were added to each well, including the standards, making a total of 200uL in each well. The plate was then incubated for ten minutes at 37°C and placed on a plate reader for analysis at 520nm.

**BCA Protein Assay**

The protein levels were analyzed in each sample from extracting the wells with NaOH. The NaOH removed any protein that adhered to the plate. The six well plates that were placed in the freezer after the isopropanol extraction were allowed to thaw to room temperature for one hour. They were extracted overnight in 1mL of .1N NaOH. 9 BCA standards also had to be prepared with concentrations from 0 to 2000μg/mL.

The BCA working reagent was also prepared with 50:1 Reagent A to B. 25uL of either standard or sample were plated in triplicates on a 96 well plate. 200uL of BCA working reagent were added to each well, making a total volume of 225uL per well. The plate was incubated at 37°C for thirty minutes and read on a plate reader at 562nm.

**Microscopy**

**Lysosome Associated Membrane Protein-1 (LAMP 1)**

As soon as the cell treatments concluded, the wells were washed two times with 1X Dulbecco’s Phosphate Buffered Saline (DPBS) and then fixed in 2.5% paraformaldehyde. All washes were 1mL. The wells were again washed with DPBS following by 10% glycerine. 100mL BSP buffer solution was made up using 1 gram of Bovine Albumin Serum in 100mL of DPBS with 0.1grams of Saponin. The wells were washed two times with BSP buffer for five minutes each wash. During the five-minute
intervals, the mice primary antibodies were made up at 10ug/mL in BSP buffer. One well of each duplicate was incubated for one hour in 500uL of the primary antibody in a moisture chamber. During the incubation, the secondary antibody was made up using alexa fluor 594 goat anti-rabbit IgG (2mg/mL) and alexa fluor 488 goat anti mouse IgG (2mg/mL). The secondary antibody was used at a working concentration of 1:2000 dilution in BSP buffer. After the first one hour incubation, the wells were washed four times in BSP buffer and all wells were incubated in the secondary antibody for one hour in a dark moisture chamber. Following the secondary antibody incubation, the wells were washed three times with DPBS, fixed in 2.5% paraformaldehyde for fifteen minutes, then washed briefly in 10% glycine. The wells were washed three times in DPBS. PPD was used as the anti-fading mounting reagent. The slide was removed from the well and a 25uL drop of PPD was placed on the slide. The coverslip from the well was inverted in the 25ul droplet. The slides were then sealed with nail polish (Ullery-Ricewick JC et al. 2009)

**LipidTOX**

The slide preparation process for LipidTOX was essentially identical to LAMP1 with a few exceptions. 1% paraformaldehyde was used instead of 2.5%. LipidTOX was used at a working concentration of 1:200 and incubated for thirty minutes (Ullery-Ricewick JC et al. 2009).

**RESULTS**

This study used biochemical analyses of changes in cellular lipids with gas liquid chromatography (GC) in addition to light microscopic observations of the location of cellular lipids. THP-1 macrophages, human macrophage cell lines, were loaded to varying degrees with cholesterol and/or triglycerides. To load cholesterol, the cells were incubated with aggregated low density lipoprotein in cell culture. For triglyceride loading, very low density lipoproteins were used. Triglyceride loading occurred either without any cholesterol loading or as a chase, after cholesterol loading. Selective inhibition of either uptake or surface hydrolysis differentiated changes produced by these two processes. The inhibition affected the levels of cholesterol and triglyceride stored in cells. Orlistat, a lipase inhibitor, was used to selectively inhibit surface hydrolysis (Guerciolini et.al, 1997). The hypothesis was that upon surface lipase inhibition, cytoplasm triglyceride levels would decrease and that, as an alternative to the surface hydrolysis, the cells would resort to receptor-mediated endocytosis to obtain triglycerides. Addition-ally, fluorescently labeled triglycerides were used to study their accumulation within the cells under varying conditions: when cells contained excess cholesterol, when cells contained normal levels of cholesterol, and in the presence and absence of various inhibitors. The two antibodies used in the light microscopy analysis were lysosome-associated membrane glycoprotein 1 (LAMP1), also known as CD107a, and LipidTOX. LipidTOX is a neutral lipid stain that was designed to identify effects on metabolism of fatty acids and lipids. It was used in this research to study the location and accumulation of lipids in the cells (HCS LipidTOX™ Deep Red Neutral Lipid Stain, 2013). The antibody identified the lipids that were stored in the lysosomes of the cells (Krzewski et. al, 2013). Since LAMP1 identified lysosomal lipids, lipid absorbed via endocytosis was distinguishable from that of the surface lipase enzyme.

In order to differentiate lysosomal lipoprotein storage from surface lipolysis, biochemical analysis was used on the treatment groups to determine the effect on triglyceride and cholesterol levels of blocking the surface lipase enzyme with Orlistat. In the presence of Orlistat, it was hypothesized that cytoplasmic triglyceride levels should decrease while lysosomal lipoprotein storage should increase. In addition, the cholesterol concentration values were of interest to determine whether triglyceride affected cholesterol metabolism. The first objective was to observe cellular VLDL accumulation in the cytoplasm using the neutral lipid staining LipidTOX. It was concluded from microscopy analysis that VLDL treatments produce an increase in cellular neutral lipid storage (Figure 1 and 2). Upon blocking surface lipolysis with Orlistat, neutral lipid staining significantly decreased.
Figure 1: DIC of 6D Control‡ 3DVLDL treatment, Differential Interface Contrast showed lipid accumulation in droplets. The second panel is 594 fluorescence labeling of 6D Control‡ 3DVLDL treatment, LipidTOX staining which confirmed a strong presence of neutral lipid.

Figure 2: DIC of 6D Control‡ 3DVLDL treatment, Differential Interface Contrast (DIC) showed lipid accumulation in droplets. The second panel is 594 fluorescence labeling of 6D Control‡ 3DVLDL treatment, LipidTOX staining which confirmed a strong presence of neutral lipid.

The increase in neutral lipid staining in Figure 1 was supported by GC analysis of triglyceride accumulation under the presence of Orlistat. Orlistat significantly decreased the triglyceride concentrations in the 3D VLDL chase treatment group. Additionally, Orlistat produced a minor increase in triglyceride in the aggregated LDL treatment and the control.
Figure 3: GC analysis of triglyceride concentration for each treatment group. Orlistat diminished cellular triglyceride concentration in VLDL chase groups. There was slight increase of triglyceride in the control and AggLDL treatments with Orlistat. The second panel was a repeat experiment of Figure 3. The increase in triglyceride concentration for the 3D VLDL chase in significantly decreased with Orlistat. Orlistat treatments of the control and AggLDL groups also showed the minor increase in triglyceride.

To analyze the buildup of lysosomal lipoprotein following an Orlistat inhibition of surface lipolysis, treatment groups were fluorescently labeled with LAMP1. Microscopy analysis indicated an increase in LAMP1 staining following the Orlistat treatment. The DIC micrograph showed lipid droplets significantly declined in the Orlistat treatment.

**LAMP1**

6D Control‡ 3DVLDL
Figure 4: DIC of treatment indicates the presence of lipid droplets. The second panel shows fluorescent labeled LAMP1 staining. Minor LAMP1 staining indicates small amounts of lysosomal lipoprotein.

6D Control‡ 3DVLDL + ORL

Figure 5: DIC of treatment indicates a decrease in the presence of lipid droplets in comparison to Figure 4. In the second panel, Fluorescent labeled LAMP1 staining indicates a drastic increase in lysosomal lipoprotein.

Orlistat treatments indicated an increase in triglyceride accumulation in the lysosome. The significance of selective inhibition is reflected in the effect of triglyceride concentration on cholesterol storage. In Figure 3, the VLDL treatment groups experienced high levels of triglycerides. 6DAggLDL followed by a 3D VLDL chase in Figure 6 resulted in significantly less cholesterol storage than 6D AggLDL without triglyceride. The inhibitory effect of triglycerides on cholesterol storage was also seen when Orlistat inhibited triglyceride surface lipolysis.

Figure 6: Cholesterol Storage

Figure 6: Cholesterol storage increased when triglyceride concentration was inhibited with Orlistat. Additionally, the 6D AggLDL ‡ 3D VLDL treatment resulted in less cholesterol storage than the absence of the triglyceride chase.
To further support the hypothesis that triglyceride presence in cells increases lysosomal metabolism of cholesterol, the percent triglyceride due to endocytosis was calculated based on whether or not the cells were preloaded with cholesterol. From this, it could be determined if the cells resorted to endocytosis to metabolize triglyceride under high concentration of cholesterol. The endocytic metabolism of triglyceride restored function of the lysosome to metabolize cholesterol.

**Figure 7: Percent Triglyceride due to Endocytosis**

The overall increase in cholesterol concentration from figure 6 shows the effect on cholesterol metabolism with the absence of triglyceride. An increase in cholesterol is attributed to the blocking of the surface lipase enzyme by Orlistat. The decrease in triglyceride leads to a dysfunctional lysosome, preventing effective cholesterol metabolism.

**Discussion**

Our data supports the hypothesis that a lack of triglycerides inhibits cholesterol metabolism. The ability to selectively inhibit intracellular triglyceride storage allowed our biochemical analysis of the presence of triglyceride and cholesterol in the cell. Figure 1 shows the LipidTOX staining of neutral lipids following a 3D VLDL treatment. Lipid droplets formed as a result of the triglyceride treatment. In Figure 2, from the DIC of the 3D VLDL+Orlistat treatment group, we were able to demonstrate a decrease in lipid droplet formation under the presence of Orlistat. As a result, Figure 2 depicts a significant reduction in LipidTOX staining. Selectively inhibiting the surface lipase enzyme in the cells with Orlistat reduced lipid droplet formation. We used this selective inhibition to analyze the resulting alternative to surface lipase inhibition: the accumulation of triglycerides in the lysosome. Our data supports the hypothesis that inhibition of surface lipolysis results in higher lysosomal lipoprotein buildup. In Figure 4, the DIC shows the presence of lipid droplets, but fluorescent labeling has little LAMP1 staining. The lack of lysosomal lipoprotein staining in the triglyceride treated group indicates that the primary form of triglyceride hydrolysis in the cell is surface lipolysis. Figure 5 has less lipid droplets due to the Orlistat treatment; however, once fluorescently labeled with LAMP1, it depicts a significant increase in lysosomal triglyceride. This supports the hypothesis that inhibiting surface lipase results in an increase of triglyceride hydrolysis in the lysosome. This supports our hypothesis of triglycerides’ effect on cholesterol metabolism.

Since we established the ability to manipulate triglyceride accumulation, GC analysis of cholesterol storage provides the link to determine the effect of cholesterol with the coexistence of triglycerides. Figure 6 depicts a significant decrease in esterified cholesterol after the VLDL chase. Treating the cells with triglycerides reduced cholesterol storage and restored lysosomal metabolism of cholesterol. After inhibiting surface lipase with Orlistat, we also observed an increase in cholesterol. We can conclude that a reduction of triglycerides in cells results in
an increase in the cells’ ability to store cholesterol. Figure 6 has an inconsistency in the data because there was not a significant difference in cholesterol storage after triglyceride treatment. This can most likely be attributed to a decrease in the cells’ ability to absorb the higher concentration of cholesterol. The repeat experiment occurred toward the end of the life of the cells, leading to a possible effect on their ability to hydrolyze AggLDL. Additionally, it can be observed in Figure 6 that in the control groups, Orlistat treatment increased cholesterol storage. This showed that even though the cells were not treated with triglycerides, blocking surface lipolysis increased cholesterol concentration. A possibility is that Orlistat prevented absorption of triglycerides in the media into the cells. In Figure 7, the percentage of endocytic metabolism was calculated before and after cholesterol accumulation. In this experiment, a 6D VLDL treatment was added to be able to confirm the altering effect of cholesterol concentration on triglyceride presence in the cell. From the data, it is apparent that the preexisting cholesterol concentration causes an increase in triglyceride metabolism through the lysosome.

The future direction of this research comprises of exploring other potential microscopy staining, including the Anti-Niemann Pick C1 antibody, which marks the intracellular transport of cholesterol. With more knowledge of the locations of cholesterol buildup, we can reach conclusions about the impact of cholesterol accumulation on triglycerides. In addition, repeat GC experiments are planned while adding treatment groups to explore the further effect of Orlistat on cholesterol storage.

REFERENCES


