THE ROLE OF AUTOPHAGY IN PHOSPHATIDYL-GLYCEROL FACILITATED CHOLESTEROL CLEARANCE FROM THE ENDOLYSOSOMAL SYSTEM OF NPC-1 DEFICIENT CELLS

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✸ ABSTRACT

Niemann Pick Type C (NPC) Disease is a rare lysosomal storage disorder in which one of the genes that codes for either the NPC-1 or NPC-2 protein is mutated, causing cell lysosomes to accumulate cholesterol and lipids. Previous studies discovered that a unique late endosomal/lysosomal phospholipid, lysobisphosphatidic acid (LPBA), is involved in cholesterol clearance from late endosomes. It has also been shown that exogenous treatment of the NPC-1 deficient cells with LBPA's precursor, phosphatidylglycerol (PG), leads to LBPA enrichment and subsequent endolysosomal cholesterol clearance. Autophagy is a mechanism of cellular clearance in the endolysomal system and we are interested to see if it is a partial route in cholesterol clearance during PG treatment of NPC-1 deficient cells. To do so, we silenced the gene that codes for an essential protein in the autophagy pathway, making the cells autophagy deficient. We then treated

the cells with PG, measured the amount of cholesterol clearance in those cells, and compared it to cells with normal autophagy. We found significantly less cholesterol clearance by PG in cells with defective autophagy, confirming that autophagy is involved as a partial route in cholesterol clearance during PG treatment, but not enough of a difference to conclude that it is a major underlying mechanism.

1   INTRODUCTION

Niemann Pick Type C (NPC) Disease results in cholesterol and lipids becoming trapped in cell lysosomes and unable to be trafficked through the cell to where they are needed.[19,26] Cholesterol accumulates in the lysosome and exits via lysosomal membrane proteins, such as NPC-1 and NPC-2, in unaffected cells. The NPC proteins are essential for transporting cholesterol through the endolysosomal system; therefore, nonfunctional NPC proteins result in an accumulation of cholesterol and secondary lipids in the late endosomal and lysosomal compartments. An NPC patient suffers from severe motor and neurological symptoms that worsen with time.[14]

The exact method of how cholesterol is transported out of the endolysosomal system is unknown,[14] but our preliminary data suggests that autophagy may be involved. Autophagy is a mechanism of cellular clearance in which damaged proteins, organelles, and membranes are transported to the endolysosomal system where they can be broken down into metabolites used by the cell.[28] It is essentially a cellular degradation and recycling process. In order for cells to perform essential biosynthetic and energy production pathways, they need basic monomers of larger molecules. Cells can obtain these monomers through the break-down and recycling of damaged organelles, proteins, and cellular membranes. These molecular degradations occur in lysosomes, which are acidic vesicles containing numerous hydrolases and found within the cell. Hydrolases are a class of proteins that break down large molecules into their monomers. Macroautophagy involves sequestering the molecules that need to be degraded into vesicles called autophago-

somes and delivering them to lysosomes for degradation,[28] as shown in FIGURE 1.

The process of autophagy begins with the process shown in FIGURE 2. Several molecules come together to form the ULK1/2-ATG13-RB1CC1 induction complex, which can develop at multiple sites throughout the cytoplasm.[5,10] Once formed, the complex can either be activated or inactivated via a molecule called the Mechanistic Target of Rapamycin Complex 1 (MTORC1). When MTORC1 is associated with the induction complex, the complex is inactive and the process of autophagy stops. When MTORC1 dissociates from the induction complex, the complex activates, resuming autophagy.[9] To summarize, the process of autophagy is inhibited when MTORC1 is associated with the ULK1/2-ATG13-RB1CC1 induction complex and resumes when MTORC1 disassociates from the induction complex.

In addition, FIGURE 2 displays how the nutrient status of the cell is involved in autophagy regulation. During nutrient starvation, there is low energy in the cell. The nutrition status therefore determines the status of autophagy, as the cell needs the monomers produced by autophagy for the energy production pathways.[9] During nutrient enrichment, there is enough energy in the cell and autophagy is not needed.

The next step of autophagy is nucleation, or formation of the membrane, which is an essential step in macroautophagy.[18] Once the membrane begins to expand, it is called a phagophore. This structure is double membranous and expands to eventually create a closed, circular vesicle.[8] The process of phagophore elongation is composed of 2 conjugated systems. The first system results in the formation of the ATG12-ATG5-ATG16L1 complex, as shown in FIGURE 3, which is needed for the second system. Once formed, the complex associates with the phagophore membrane.[15,16] The second system is shown in FIGURE 4 and ultimately allows the phagophore membrane to elongate and move locations within the cell.[18] One step in this process is facilitated by the ATG12-ATG5-ATG16L1 complex formed in the first system. As the phagophore membrane expands, it curves around its target molecules,
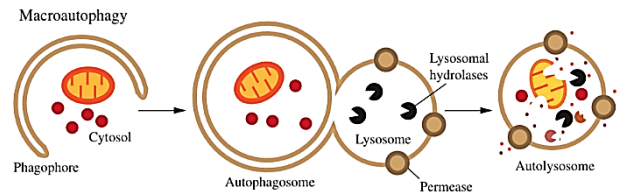

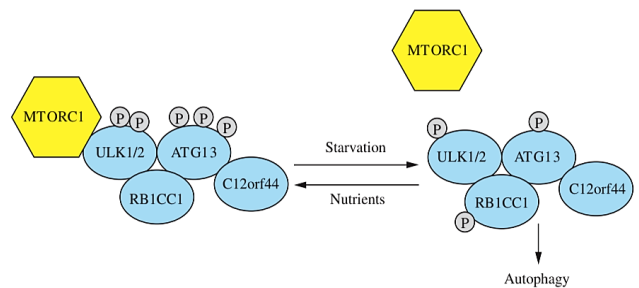**FIGURE 1:** *Overview of macroautophagy in mammalian cells. Figure obtained from* [18].

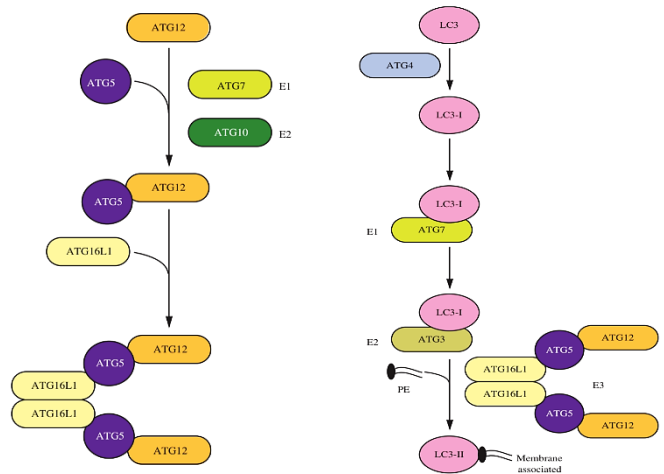**FIGURE 2:** *Activation of the induction complex during a nutrient starved cellular state. Figure obtained from* [18].

FIGURE 3: (LEFT) *ATG12–ATG5-ATG16L1 conjugation complex. Figure obtained from* [18].

FIGURE 4: (RIGHT) *ATG8/LC3 conjugation system. Figure obtained from* [18].

known as cargo, and keeps adjusting to wrap around the cargo.[18] When it has fully wrapped around the cargo, the ends connect, forming a closed double membranous vesicle known as an autophagosome.[18]

The autophagosome will move toward the lysosome via the cell's microtubules.[17] Once at the lysosome, the autophagosome's outer membrane will fuse with the lysosomal membrane, creating an autolysosome. The autophagosome may also fuse with endosomes, in both early and late stages, before fusing with the lysosome.[2,25] The last step of autophagy is referred to as autophagic flux where degradation within the autolysosome begins when the inner membrane is disrupted by ATG15.[7,24] Once the inner membrane is disrupted, the cargo that the autophagosome carried is exposed to the lysosome's acidic environment and hydrolases that degrade molecules into their basic monomers. These metabolites are then exported from the lysosome into the cell via transporter proteins on the lysosomal membrane.[29] Different metabolites are transported out by different receptors. NPC-1 and NPC-2 proteins are examples of these receptors used in the transport of cholesterol out of the lysosome.[20,27]

Niemann Pick C (NPC) Disease is a type of lysosomal storage disorder, which is a category of disorders usually caused by a mutation in a gene that codes for a lysosomal transport protein.[21] Several studies have shown that autophagy is blocked or dysfunctional in most lysosomal storage disorders because of impaired fusion between the autophagosome and the lysosome.[13,22,23] This block is thought to be caused by abnormal cholesterol accumulation in the lysosome.[1] Therefore, in NPC disease, since one of the NPC proteins is nonfunctional, cholesterol will be trapped in the lysosomes and autophagy will be blocked due to an inability of the autophagosome to fuse with cholesterol-filled lysosomes.

It was found that cholesterol clearance depends on the interaction of a region of the NPC-2 protein with a lipid called lysobisphosphatidic acid, or LPBA.[6] In patients with NPC-1 disease, meaning their NPC-1 protein is dysfunctional, cholesterol clearance is still affected. This is because the limiting membrane of a lysosome is covered in a sugar coat called glycocalyx and it is believed that the luminal domains of the NPC-1 protein are required for cholesterol to be able to penetrate through the glycocalyx and exit the lysosome.[11,12] Increasing the LBPA levels in NPC-1 deficient cells have been found to result in a significant amount of cholesterol clearance because the LBPA can still interact with the NPC-2 protein.[6] Exogenous treatment of LBPA's precursor phosphatidylglycerol, or PG, also results in cholesterol clearance because the PG can go through its biosynthetic pathway to make LBPA.[13]

Our preliminary data shows that PG treatment of NPC-1 deficient cells facilitates autophagosome fusion with the lysosome and subsequent autophagic flux. Since NPC-1 deficient cells block autophagy and PG helps it resume, we are interested in seeing if autolysosome formation and autophagic flux are involved in cholesterol clearance from late endosomes when these cells are treated with PG. To determine whether PG/LBPA-induced autophagic flux and cholesterol clearance are directly linked, we blocked autophagy by silencing the gene that codes for the ATG12 protein. ATG12 is essential to autophagy because it is part of the ATG12-ATG5-ATG16L1 complex that binds ATG8/LC3 to the membrane, an essential process in phagophore elongation. Autophagy can be blocked by silencing the genes of a number of essential proteins to the process, but we will only focus on the gene that codes for ATG12. After making the autophagy knockdown cell line and the control cell line, we treated the cells with PG and measured the subsequent cholesterol accumulation to see if the cells with blocked autophagy still cleared cholesterol to the same extent. If autophagy is involved in cholesterol clearance during PG treatment, the cells that received the PG treatment but had autophagy blocked would have less cholesterol clearance than cells that received the PG treatment and had functional autophagy.

2   Methodology

NPC-1 deficient cells were seeded in 12-well plates on coverslips with antibiotic-free growth medium supplemented with FBS (fetal bovine serum), which contains nutrients that help the cells grow and divide. The cells were NPC-1 deficient either be-

cause they were fibroblast cells taken from patients with NPC-1 disease or because they were NPC-1 knocked out HeLa cells, meaning the gene that codes for the NPC-1 protein was silenced. These HeLa cells are true NPC-1 -/-, meaning NPC-1 will be 100% knocked-out.

After 24 hours, the cells were at confluency, or 0.5 x 10^6 cells per plate, and we washed the cells with shRNA Transfection Medium. We then made 2 cell lines: an autophagy knockout line and a control line. The cells for the autophagy knockout line were transduced with ATG12 shRNA lentiviral particles and the cells for the control line were transduced with scrambled control shRNA lentiviral particles. The cells of both lines were transduced with shRNA lentiviral particles in concentrations of 1x10^5 infectious units of virus (IFU). The ATG12 shRNA encoded for a sequence that degraded mRNA in the cell that coded for the ATG12 protein. Autophagy was blocked because these cells could not make the ATG12 protein. The scrambled control shRNA encoded for a sequence that did not degrade any known cellular mRNA. Since these cells could express all of their normal proteins except NPC-1, autophagy was not blocked. Knockdown shRNA did not get rid of 100% of the target mRNA, and preliminary experiments showed that autophagy would be reduced but not completely blocked.

After 48 hours of incubation, the old medium was removed and 2 µg/mL antibiotic puromycin medium was added to all well. Cells transfected with the virus particles would have a gene that make the cells resistant to puromycin, while cells that were not transfected would be killed by the antibiotic. Cells were then incubated for 3 days, after which all stable cells that remained alive were known to be the ones that were transfected.

Each cell line had a control group and a treatment group, with a total of 48 wells. We treated both treatment groups with 100µM PG and let the cells incubate. After 48 hours we washed all cells using phosphate buffer saline (PBS) and fixed them to the coverslips using a 4% paraformaldehyde solution. We then stained the cells on the coverslips with cholesterol-binding polyene antibiotic filipin III and a nuclear dye (sytox green). Each of the 48 coverslips

contained about 50-100 fibroblast cells. Images were taken with the epifluorescent Revolve Microscope at 40x magnification. Fluorescent intensities of the subcellular structures stained with filipin were analyzed using Image J software as described by.[11]

3 RESULTS

Fluorescent microscopy detects the intensity of filipin from the coverslips on which the cells were fixed (FIGURE 5). Filipin binds to cholesterol; the higher the filipin intensity, the higher the cholesterol levels in the cells. The results of the fluorescent microscopy are shown in FIGURE 6.

Scr Control is a control group that is NPC-1 deficient only. Scr+PG is a PG-treated group that is NPC-1 deficient only. Both of these groups came from the control cell line that received the scrambled control shRNA. ATG12 KD Control is a control group that is both NPC-1 deficient and has autophagy blocked. ATG12 KD + PG is a PG-treated group that is NPC-1 deficient and has autophagy blocked. Both of these groups have autophagy blocked because they came from the autophagy knockout cell line that received the ATG12 shRNA. The untreated control groups provide the baseline filipin intensity for each cell line.

FIGURE 6 shows that there is a statistically significant difference in cholesterol content between the Scr control and Scr+PG groups, with Scr+PG having less cholesterol. This is in agreement with the previous finding that PG supplementation increases LBPA levels and thus decreases cholesterol content.[14] The red bracket in FIGURE 6 shows that ATG12 KD+PG has significantly more cholesterol than Scr+PG. This shows that autophagy is a partial route in cholesterol clearance during PG treatment, since the PG treatment was less effective when autophagy was blocked. Lastly, FIGURE 6 shows that ATG12 KD+PG has significantly less cholesterol than ATG KD Control. This indicates that autophagy is not the major underlying mechanism for PG/LBPA-induced cholesterol efflux since PG treatment had an effect even when autophagy was blocked. This is also suggested by the fact that there is only 15% more cholesterol in ATG12 KD+PG than in Scr+PG; there would be closer to 100% more cholesterol in ATG12
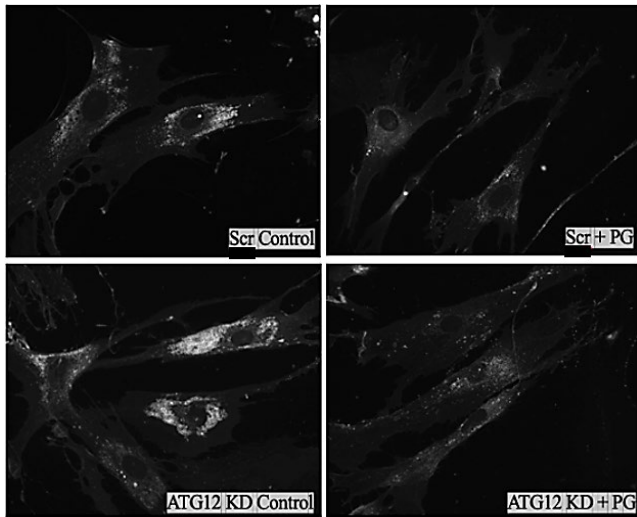
FIGURE 5: *Sample of images of NPC-1 fibroblast cells in each condition, filipin stained.*
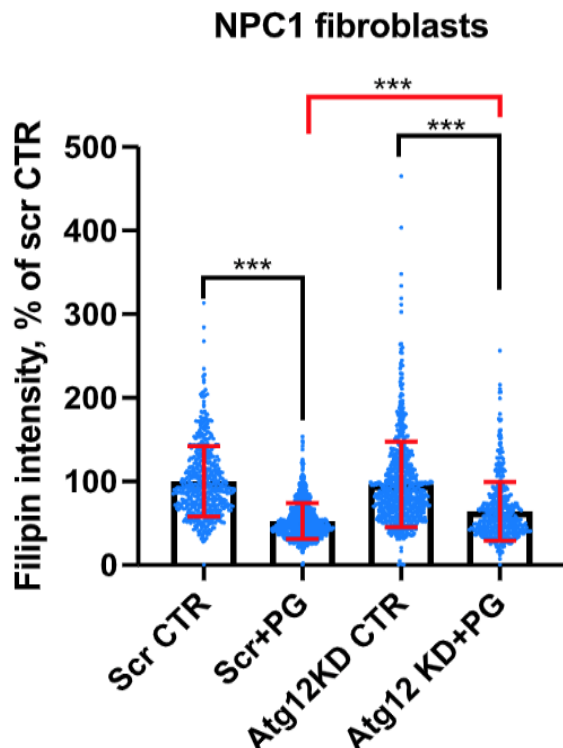

FIGURE 6: *Quantification of filipin intensity in NPC1-deficient fibroblasts stably transduced with lentiviral non silencing control shRNA (Scr) or shRNA targeting Atg12 and selected for stable shRNA expression. **N** > 500 cells/condition. Data from 2 independent experiments. ∗∗∗ signifies a **p** value < 0.001.*

KD+PG if autophagy was a major route in cholesterol clearance. The statistical tests were t-tests using a significance level of 0.01 and all three tests produced a p value of less than 0.001, as indicated by the asterisks.

4   DISCUSSION

The statistically significant results between Scr+PG and ATG12 KD+PG indicate that autophagy is blocked in NPC disease due to an inability of the autophagosome to fuse with the lysosome and this impaired ability is restored by PG/LBPA enrichment in NPC-1 cells, ince the inner-lysosomal portion of the NPC-1 protein is thought to be required for cholesterol to penetrate through the glycocalyx that coats the limiting lysosomal membrane, enhanced autolysosome formation during PG/LBPA enrichment could potentially provide a route for cholesterol egress that bypasses the glycocalyx.[11,12,13,22,23] We hypothesize that the glycocalyx of the lysosome may become weakened when the autophagosome fuses with the lysosome, allowing cholesterol to more readily reach the limiting membrane of the lysosome, and therefore more readily exit the compartment.

NPC-1 disease shares many similarities with Alzheimer's disease.[3] Increased endogenous LBPA has been reported in both NPC-1[6] and Alzheimer's disease,[4] where such an increase might be a compensatory homeostatic cellular response to eliminate toxic deposits. Therefore, PG/LBPA enrichment has great potential for the treatment of NPC-1 disease and potentially other lysosomal storage disorders and neurological diseases with autophagy defects, such as Parkinson's or Alzheimer's disease. It is important to understand all processes involved in cholesterol clearance during the treatment, including autophagy. In future work, to understand the extent of autophagy's role in cholesterol clearance during PG treatment, we will block autophagy by silencing other molecules, such as ATG5. There are many ways that autophagy can be blocked because there are many essential proteins to the process that can be silenced. Silencing some genes for autophagy proteins may result in a more complete knockdown than silencing other genes.

Patients with NPC-1 disease develop detrimental neurological symptoms because their cells accumulate cholesterol in the endolysosomal compartment and are unable to clear the cholesterol from that compartment. LBPA clears cholesterol from the endolysosomal compartment, which indicates that potential therapies to prevent the neurological symptoms may be found in interventions that increase LBPA, such as PG treatment. Uncovering the full process of PG-facilitated clearance of cholesterol will aid in manipulating LBPA levels for potential therapeutic benefits in patients with NPC disease, other lysosomal storage disorders, and neurological diseases ∎

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My name is Tamara Allada and I am a 2021 Rutgers graduate and the main author of this paper. I spent my final two years of college working with Dr. Judith Storch and Dr. Olga Ilnytska in the Rutgers Department of Nutritional Sciences. Our lab's main area of study is the method of intracellular cholesterol transport, as the exact pathway in which cholesterol is trafficked through a cell is currently unknown. There are many lysosomal storage disorders, such as Niemman Pick C Disease, that cause cholesterol to become trapped in a compartment of the cell and unable to be trafficked, resulting in severe neurological defects. Knowing the exact pathway of intracellular cholesterol transport would aid greatly in the search for a treatment to such disorders.