ABSTRACT

Alcoholic and nonalcoholic fatty liver disease is projected to be the most common cause of liver disease in developing countries. The main significant risk factors are obesity, diabetes mellitus type 2, cardiovascular disease, and dyslipidemia. Louisiana is ranked seventh in liver cancer diagnoses and ranked sixth in the leading cause of death. Recent findings indicated that multifaceted stress response due to the accumulation of fatty acids from the diet is the driving force of disease progression. We sought to study multifaceted integrated stress response (ISR) in liver cells cultured with saturated fatty acids. Understanding the process that ISR takes to either induce or inhibit autophagy, self-eating machinery, in strongly permissive HUH 7.5 cells is vital when treating liver abnormalities. The major protein kinase, P-EIF2 alpha, was the targeted factor contributing the most to autophagy due to its functional link to the endoplasmic reticulum, mitochondria, and cellular membrane by further assessment using the inductive drug, Sephin 1. HUH, 7.5 liver cells are treated with increasing amounts of palmitic acid for 24 hours in DMEM with 10% FBS. ISR activated after substantial cellular damage leading to autophagy impairment. The cell culture was assessed for lipid accumulation, and the expression of PKR, IRE1 alpha, PERK, ATF6, P-EIF2 alpha, HRI, MTORC1, GCN2, P62, and LC3B was achieved by immunoblot analysis. Membrane fluidity PKR, lysosomal MTORC1, and protein synthesis GCN2 activated to elicit an integral response to the ISR pathway. Endoplasmic reticulum protein kinases induced in response to UPR activation lead to an integration of the P-EIF2 alpha pathway. Mitochondrial stress heme regulated inhibitor proliferated to provoke an activation in the significant protein kinase leading to autophagy impairment. The P-EIF2 alpha kinase invoked autophagic deficiency even when dephosphorylation was prevented by Sephin 1 drug treatment. ISR constrained autophagy in the liver-derived cell line due to the accumulation of the toxic saturated fatty acid.

Keywords: palmitate, autophagy, fatty liver disease, integrated stress response, Sephin 1
ROLE OF INTEGRATED STRESS RESPONSE IN THE PROGRESSION OF LIVER DISEASE

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I. Chapter 1: Introduction

1. Overview

The molecular mechanism of how fatty acids induce or inhibit autophagy during the proliferation of liver cells is the study's premise. Palmitate, the toxic saturated fatty acid, controls and modifies various cellular physiological processes such as inflammation or membrane fluidity (1). A diet enriched in saturated fatty acids will likely promote hepatic inflammation and elevated cellular stress, known as the integrated stress response. When liver cells are incubated with saturated fatty acids, the integrated stress response is expected to cause organelles such as the endoplasmic reticulum, mitochondria, and the lysosome to combat the toxic effects (2). It is proposed that the effect of integrated cellular stress will change membrane fluidity, further promoting an upstream of oncogenic activations and inhibit translation of proteins. To combat integrated stress, slow the progression of fatty liver disease and promote cell survival, a cure or treatment would be the best approach and hopefully lead to an induction of autophagy. Before developing the therapeutic measures, a complete understanding of integrated stress response in liver disease progression is required.

This overall analysis led to the broad question of how do fatty acids directly modulate autophagy? Through which pathway, or what inducer, can there be a guarantee of autophagy inhibition. Will the proposed P-EIF2 alpha pathway be activated when treated with aggregate doses of palmitate? What effect will the activated P-EIF2 alpha kinase have on the modulation of autophagy? If this study yields accurate results and further research is conducted, what will occur if the phosphorylation of P-EIF2 alpha was induced or inhibited by drugs, and what effect will it unswervingly have on autophagy? Once the study development is recognized, how can targeted therapies be implemented to treat and eventually reduce liver abnormalities entirely? This project aimed to explore the general question and further understand the role of the phospho-EIF2 alpha pathway on the inhibition of autophagy. Hopefully, this project's future direction will use the results of developing immune response mechanisms or biomarkers dependent on P-EIF2 alpha to reduce hepatic inflammation due to fatty liver and progressively resolve liver disease progression.
2. Fatty Liver Disease and Lifestyle Contributions

The fatty liver gradually develops into a spectrum of abnormalities that range from nonalcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH) to fibrosis, cirrhosis, and eventually hepatocellular carcinoma (liver cancer). It is characterized by excessive lipid accumulation and metabolic disorder of liver cells termed hepatocytes (3). HCV is a significant public health problem that infects the liver, and it is currently prevalent in over 3% of the world population (4). Hepatitis C infection interacts with other liver abnormalities using host and viral factors, including mediators of metabolic syndrome, inflammatory cytokines, oxidative stress, apoptosis, hepatic stellate cell activation to influence liver disease progression (5). Fatty liver is very common in HCV-infected patients, including the prevalence of steatosis and fibrosis (6). Steatosis (NASH), abnormal retention of cellular lipids, paired with NAFLD in obese patients, has also been shown to promote oxidative damage, cellular impairment, and other host and viral factors that deteriorate liver injury (6). To further comprehend the influencing factors of NAFLD, lifestyle contributions should be incorporated as determining factors.

The prevalence of adults diagnosed with chronic liver disease in the United States is currently at 4.5 million, 1.8% of the adult population (7); moreover, there is a substantial racial disparity with liver disease. Within the racial spectrum in the U.S., African Americans have twice the prevalence of HCV and progressively develop liver cancer faster than any other race. The modern-day lifestyle and diet contribute to the progression of liver disease among children and adults. Obesity has increased the prevalence of nonalcoholic fatty liver disease (NAFLD), including type 2 diabetes, hypertension, stroke, and cancer, which is a significant cause of chronic fatty liver disease worldwide (8-9). It is still being studied whether obesity can cause the same kind of liver damage as alcohol; furthermore, fatty liver affects between 70% to 90% of individuals battling obesity and diabetes (10). Based on current health data in the United States, non-Hispanic black adults have the highest prevalence of obesity, followed by Hispanic adults consecutively at 49.6% and 44.8% (7). Although further research is still being conducted to understand the correlation between lifestyle contributions and racial groups, the advancement of how intrahepatic fat impairs the cellular environment is well understood.

3. Understanding Intrahepatic Fatty Acid Disruption
The accumulation of intrahepatic fat results in liver inflammation termed steatohepatitis and results in further liver damage leading to liver fibrosis and cirrhosis (10). Steatohepatitis develops when the fatty acid input rate is more than the output that the liver can maintain (8). Fatty acids (FA) are derived from triglycerides or phospholipids as a source of energy obtained through human processing, which causes membrane disruption and eventually causes a stressed cellular disturbance. Within the liver, an increasingly stressed environment leads to abnormal lipid metabolism and decreased fatty acid oxidation. FA oxidation is a mechanism where FA is broken down to produce energy within the mitochondria, peroxisome, or the endoplasmic reticulum. For mitochondrial FA oxidation, Acetyl-CoA, a precursor for fatty acid production, will guide the lipid modification of proteins and produce triglycerides (11). The nutrient metabolizer, hepatocytes, transforms excess glucose derived from our nutritional diet into FA by de novo synthesis within the cytoplasm or mitochondria. It is essential to know that deregulation of the de novo FA synthesis will result in cellular fatty acid accumulation, impaired signal transduction, and gene expression (11). An increase of de novo FA synthesis is a progenitor of cancer pathogenesis (11). The de novo FA synthesis being overworked or reduced appears to lead to problems, which poses a high fat or low-fat diet aids or dysregulates the de novo FA synthesis.

Due to the stressed environment, hepatocytes are overworked to uptake the blood's fatty acids and assemble them with glycerol to produce triglycerides. Our modern diet paired with a lack of physical activity leads to nonalcoholic fatty liver and steatosis due to the liver's abnormal accumulation of fat. Individuals battling pre-existing conditions such as insulin resistance, diabetes, and obesity, have a higher risk of progressing to nonalcoholic Steatohepatitis (NASH). In individuals with NASH, liver homeostasis is impaired due to the accumulation of toxic lipids that activate immune responses contributing to local inflammation of hepatic tissue. The pathological environment provokes hepatocyte damage and leads the liver to a state of ballooning. Hepatocytes suffering apoptosis and inflammation lead to the release of signaling molecules for hepatic stellate cells activation to secrete collagen fibers that form scar tissue leading to hepatic fibrosis. NASH can evolve into cirrhosis and eventually hepatocellular carcinoma, while it increases the risk of cardiovascular events due to NASH patients' lipid profile.
The fundamental treatment for fatty liver disease includes lifestyle modifications such as diet changes to promote weight loss and increased physical activity (12). Researchers have found that exercise paired with calorie restriction can increase lipolysis to consume liver triglycerides and eliminate steatosis (9). An aggressive reduction of sugars, starches, and simple carbohydrates have been listed as progressive changes that will significantly reduce fatty liver disease. It is predicted that NASH will become the leading cause of liver transplantation within the next few years due to the increasing obesity epidemic. This symptomless condition is the second most common cause of cancer; therefore, researchers continue to study the cellular pathways to address and treat this growing problem.

4. Integrated Stress Response Pathway and Signals

The physiological changes and pathological conditions that occur within specific eukaryotic cells activate integrated stress response to signal the cellular disturbance back into a homeostatic state. The stressors can be extracellular or intracellular disturbances such as nutrient or growth factor deprivation, hypoxia, ROS, DNA damage, damaged organelles, temperature, and pH (13). The stress responses could be unfolded protein responses, oxidative responses, and DNA damage responses combined with the general approach for all stressors, integrated stress response (ISR). What are the responses to alleviate integrated stress response? Depending on the stressor, homeostasis, DNA repair, and protein folding denaturation are necessary adaptive measures to combat the cellular stress response mediators. The stress intensity can either lead to apoptosis, necrosis, or autophagy, a lysosomal degradation pathway (4).

Autophagy involves the sequestration of cytoplasmic components within autophagosomes. It is simply an intracellular self-degradation and protective process that focuses on cellular cleansing and recycling of cellular nutrients. A few of the core autophagy pathway components are controlled by cellular stress signals (13). There are three significant autophagy types: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). Macroautophagy, known as autophagy, is the essential self-eating process performed to promote intracellular components' degradation (14). Microautophagy causes direct engulfment of nearby cytosolic materials or protein cargoes into endosomes or lysosomes via direct inward membrane rearrangement (4). CMA is direct translocation across the lysosomal membrane mediated by chaperone proteins. The autophagy pathway begins with phagophores' formation, an isolation membrane
from many sources such as the endoplasmic reticulum, mitochondrial membrane, and lipid bilayer (13).
The closure of the phagophore membranes form double-membrane autophagosomes with a lysosome creates an autolysosome where contents are degraded (4). The autophagy pathway is a catabolic process that generates energy through the lysosomal degradation of cytoplasmic organelles in the autolysosomes (4). It is through lysosomes that autophagy can uptake and get rid of cellular stressors. Lysosomal degradation will be studied through the MTORC1 (Mammalian target of rapamycin complex 1) pathway that senses and integrates environmental and nutritional cues such as cellular stress (Figure 2). MTORC1 translocates to the lysosome to promote autophagy and lysosome biogenesis, lipid metabolism and lipid synthesis, cell growth, mitochondrial metabolism, mRNA translation, and adipogenesis (15).

When endoplasmic reticulum stress, mitochondrial stress, viral infection, heme deprivation, and amino acid deprivation serve as the stressors, the P-EIF2 alpha kinase acts as a combination dependency kinase to invoke protein synthesis and homeostasis when induced. P-EIF2 alpha is a eukaryotic initiation factor (2) that downregulates protein synthesis when exposed to stress conditions (16). The phosphorylation of EIF2 alpha will lead to adaptive responses such as a decrease of cytokine production and energy consumption, reduced protein overload, and autophagy to promote cellular homeostasis. On the molecular level, the phosphorylation of the EIF2 alpha measures ISR by promoting cap-independent mRNA translation (4).

Figure 1: Integrated stress response promotes cap-independent translation.
Stress kinases prevent the EIF2 alpha complex from forming due to the integral connections.
During eukaryotic translation, cap-independent translation occurs when the cell is under immense stress and trying to survive with tools provided from the minimal enzyme activity maintained. The internal ribosome entry sites (IRESs) will allow the ribosome to enter the mRNA. The effect of palmitate on cellular proteins will be assessed by the GCN2, an ISR activator for adaptation to amino acid protein starvation (17). GCN2 is a general control non-depressible 2 activated by uncharged tRNAs in amino acid starved cells (13). The termination of ISR will require a complete restoration of protein synthesis and normal cell functioning. In Figure 1, ATF4 was found to control integrated stress response; therefore, finding other kinases to aid in the control is the next step for researchers and the premise for this study.

5. Fatty Acid action within the cellular membrane

i. Overview of Membrane Fluidity

Cholesterol, unsaturated FA, and saturated FA are the primary regulators of membrane fluidity observed in this study. Cholesterol acts as a buffer in maintaining normal fluidity. Cholesterol can be inserted anywhere in the bilayer, leading to clusters, increasing the distance between phospholipids, and increasing fluidity. Saturated fatty acids have no double bonds, and all the carbons are occupied by hydrogens, while unsaturated FA has double bonds resulting in kinks. The kinks in unsaturated FA can push molecules away to help maintain the fluidity, which is why unsaturated acids do not necessarily impair the cell membrane. Saturated FA draws molecules to itself and exerts pressure on the intermolecular forces, leading to compression and decreased membrane fluidity. It is proposed that the accumulation of palmitate, a saturated fatty acid, will intracellularly impair membrane fluidity and induce the activation of numerous stress response pathways resulting in the inhibition of autophagy.

ii. Overview of Saturated FA influence within the cell

Membrane fluidity can be impacted by a change in temperature and degrees of unsaturation, in addition to cholesterol. Therefore, due to saturated fatty acids' characteristics, an increase in saturated fatty acid content should theoretically decrease membrane fluidity. Researchers have studied that there is a possible modification on the structure of the cell lipid membrane that induces a change in membrane fluidity when cells are exposed to specific fatty acids (18). Molecularly a sudden increase of palmitate in the cellular environment will result in the introduction of toxic metabolites such as ceramide.
(Sphingolipids) and lipopolysaccharide (LPS) that will later cause activation in membrane fluidity. The stimulation of pro-inflammatory cytokines induces increasing ceramide production due to an influx of saturated fatty acids in the cell (19). To reduce the toxic metabolites due to lipid overload in the cellular environment, autophagy should be induced to get rid of the overload by using the extracellular vesicles provided by palmitate within the lipid bilayer for transportation. Ceramides are vital for the lipid structure, with cell signaling and inducing oxidative stress and liver inflammation (20). Ceramide production has also been found to be related to obesity, and not just with palmitate substrates alone and insulin resistance (21-22).

Saturated fatty acids influence the fluidity of the membrane due to increased integrated stress, which will eventually lead to other stress pathways in different organelles. The stress response kinase that represents and monitors membrane fluidity is PKR, a double-stranded protein kinase R (RNA activated) that plays a role in signal transduction and cell proliferation. PKR is highly expressed in non-hematopoietic tissues such as the liver, and the protein kinase correlates with tumor progression (23). It is known as an inducer of autophagy during viral infection (24) that works to impair eukaryotic initiation factors and eventually inhibit translation resulting in the shutdown of cellular and viral protein synthesis (23). This commonly occurs in stressful environments, resulting in activation of P-EIF2 alpha kinases, including PKR, PERK, GCN2, HRI, to mention a few (24). The mentioned kinases are vital for autophagy induced by diverse stressful conditions (24). A high concentration of palmitate interacts with PKR due to the disruptions that the fatty acid caused in the signaling pathway, resulting in the induction of autophagy (25). Saturated fatty acids will induce stress kinases in numerous ways that will eventually signal autophagy to decrease cell damage, inflammation, and other cellular disturbances for the return to homeostasis. Membrane fluidity is equally vital in the cellular endoplasmic reticulum, mitochondria, proteins, and lysosomes.

iii. Endoplasmic Reticulum Synthesis

The extended membrane system on the endoplasmic reticulum is the largest cellular organelle in many cells. It can be segregated into many compartments, including the rough endoplasmic reticulum coated with ribosome performing translation and the smooth endoplasmic reticulum with few ribosomes.
Both components can vary from cell type, and in the liver cells, the endoplasmic reticulum plays a vital role in lipid synthesis. The cells that synthesize proteins will have more rough ER, while other cell types synthesizing steroidal hormones will have more smooth ER. Each component can vary in cells depending on what stimulates the cell, such as a b cell will activate rough ER to generate antibodies while many cells smooth ER if need to detoxify alcohol, drugs, and toxins. The ER performs functions such as translating proteins into the rough ER, and it stores calcium in cells used for signaling for function. Autophagy, a cellular damage repair mechanism, responds that cells obtain if calcium levels are low or apoptosis, programmed cell death if calcium levels are too high. The ER is the site of synthesis of the many lipid molecules incorporated into cellular organelles' membranes. The various compartments in the ER can change their environment, concentrations, oxidizing, or reducing nature to accomplish specific functions.

ER is now acknowledged as a highly vital organelle that can poise cells for survival or death based on the cellular stress factors present. In essence, ER is exceptionally responsive to stressors that exhaust cellular energy or Ca2+ levels and modify the ER luminal redox state. Physiological and pathological insults, such as the manifestation of reactive oxygen species (ROS), ischemia/reperfusion, perturbations in Ca2+ homeostasis, and release of inflammatory cytokines and toxins, can all lead to a buildup of defective misfolded and unfolded proteins in the ER lumen, a condition referred to as ER stress (26). The effects of the lipid membrane, in addition to the effects of palmitate, can play a role in endoplasmic reticulum stress resulting in unfolded protein responses that leads to lipotoxicity, also known as cell death (27). The resulting apoptotic regulation will be observed along with whether autophagy was induced or inhibited.

One study found that palmitate induces endoplasmic reticulum stress and leads to an autophagic flux to unfolded proteins to promote homeostasis's protein function (28). Increased ER stress will lead to an elevation in the PERK, IRE1, and ATF6 kinases, which lead to either inflammation or induce autophagy. Reviewing the link between ER stress, autophagy induction, and inflammation in liver diseases will be beneficial when it comes to the time for finding therapeutic approaches for treatment or biomarkers. The expressions that lead to inflammation or autophagy can also be referred to as studying the apoptotic bodies that form and proliferate the cell's environment.
The activation of stress on the endoplasmic reticulum will be assessed by IRE1 (Serine/threonine-protein kinase/endoribonuclease), PERK (Protein endoplasmic reticulum kinase), and ATF6 (Activating transcription factor 6) in this study (Figure 2). IRE1 functional properties included acting as a critical sensor and upstream signal for ER unfolded protein response (UPR) in stressed cells (29). PERK is activated when cells are exposed to ER stress induced by UPR triggers, which also activates ATF6, promoting its translocation to the nucleus to function as an active transcription factor (30). PERK contributes to regulating protein translation and cell adaptation to ER stress that induces phosphorylation of EIF2 alpha after initiation of ER stress (30). IRE1 and PERK sense the stress signals caused by a disturbance and convey them to downstream effectors by their enzymatic activities that regulate fundamental cell functions (31-32). IRE1 and ATF6 are implicated in the transcriptional arm of the UPR, resulting in the induction of ER chaperones to remedy protein misfolding (30).

iv. Mitochondria Synthesis

The mitochondria provide energy to serve as the powerhouse of the human body. It takes fat, sugar, and protein from our food intake and combines it with oxygen to convert it into chemical energy for our cells and tissues called ATP, adenosine triphosphate. Cellular respiration is the process that converts our food intake into ATP; Inside the mitochondria, there are two membranes, an outer and an inner, that are important for the process of cellular respiration and are sites for oxidative phosphorylation in the electron transport chain. Cells that use more energy contain more mitochondria, such as the muscle or liver. Mitochondria has its DNA, genetic information, differing from nuclear DNA that determines our physical characteristics. Both types of DNA must be healthy for the mitochondrial to function effectively; furthermore, the mitochondria are more prone to DNA damage. Faults in either DNA type can cause the mitochondria to stop working correctly, preventing them from converting the fuel intake into energy. If there is a disturbance, our cells will run out of energy and potentially fail. A defect in the mitochondria does contribute to the progression of liver disease and can become fatal. It is still being studied how mitochondrial stress is related to the integrated stress response. It is understood that mitochondria are essential organelles in eukaryotic cells that play a significant role in cellular homeostasis and signaling (33). The effects on altered protein homeostasis due to mitochondrial dysfunction affect protein folding.
compartmentalized in eukaryotic cells. The stress response and UPR signaling pathways transcriptionally regulate organelle-specific molecular chaperones to reduce protein folding (34). The failure of mitochondrial proteins properly folding can cause electron transport chain defects and an accumulation of ROS (34). High levels of ROS lead to DNA, lipid, and protein damage; therefore, the removal of damaged mitochondria is known as Mitophagy to prevent the accumulation of ROS for mitochondrial quality control (13). Heme, a component of iron, is synthesized in the mitochondria; its metabolism leads to mitochondrial decay, iron accumulation, and stress proliferation (33). Mitochondrial defects trigger the integrated stress response resulting in heme depletion to form during mitochondrial stress. Mitochondrial dysfunction is signaled by HR1 (heme-regulated inhibitor), which inhibits protein synthesis as a reaction to integrated stress conditions (33). HR1 controls translation by phosphorylating EIF2 alpha to signal mitochondria stress to integrated stress response (Figure 2).
Figure 2: Proposal of ISR Inhibition of Autophagy  The increasing treatment of palmitate in HUH 7.5 cells was hypothesized and found to proliferate kinases of membrane fluidity, endoplasmic reticulum, mitochondria, lysosomes, and proteins to activate P-EIF2 alpha for an overall inhibition of autophagy due to ISR induction.
II. Chapter 2: Palmitic Acid inhibition of Cellular Autophagy

1. Overview

The purpose of this chapter is to evaluate the effect of palmitate inhibiting autophagy in stressed HUH 7.5 cells when treated with increasing doses. The cells were cultured in 6-well plates and were treated for 24-hours with the following doses: 0.05 uM, 0.1 uM, 0.25 uM, 0.5 uM, and 1 uM. The obtained cell pellet was used to assess fatty acid accumulation by immunofluorescence and immunoblot protein concentration. The specific aim of this thesis is to understand the role of integrated stress utilizing the P-EIF2 alpha pathway as a possible mechanistic approach to further understand how autophagy is inhibited.

2. Materials and Methods

i. Cell Culture, Media, and Growth Conditions

The strongly permissive immortal life Huh-7.5 cell line was obtained from the laboratory of Charles M. Rice (Rockefeller University, New York). The cell line was cultured at 37 degrees celsius with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2mN 1-glutamine, sodium pyruvate, nonessential amino acids, 100U/mL, 100ug/ml of streptomycin, and 10% fetal voice serum (FBS). The growth media (GIBCO) was filtered and preserved to prevent contamination. During trypsinization methods for collecting cells, the maintained trypsin-ETDA was obtained from Gibco, likewise the Phosphate-buffer saline (PBS). The cell line was cultured in a six-well culture plate using the pre-made growth media. Each passage involved aspiration of DMEM Media, 1 ml PBS swirl wash, 400ul trypsin incubation for 3-4 minutes in the 37-degree celsius. 500ul of DMEM Media is added to the free-flowing cells transfer to a new six-well plate after centrifugation and resuspension in a fresh growth medium. The cells were cared for with diligence in the humidified incubator, and experiments were conducted at 80-100% confluency.
ii. Lipid Accumulation

The Huh 7.5 cells were first treated with control, 0.05uM, 0.10uM, 0.25um, 0.50uM, and 1.0uM of 10mM palmitic acid for 72 hours and incubated. Once the treatment time elapsed, the DMEM plus 10% FBS media was aspirated. The cells were washed twice with Phosphate-buffered saline (PBS). Fixation with Formalin 10% and 60% isopropanol began the cell staining process. Sections were washed five times in purified water (GIBCO). Selective detection of neutral lipids was activated by the evenly coated Oil Red O solution (from filtered three parts Oil Red stock Solution and two parts water). After discarding the incubated Oil Red working solution, DAPI (4' 6-DIAMIDINO-2-PHENYLINDOLE) was administered before visualization analysis showed the lipid accumulation appear red, while the nuclei appear blue.

iii. Immunoblot Analysis

The analysis of the protein concentration occurred immediately after the cells were treated with palmitate for 24 hours. For the drug treatment analysis, after 24-hour treatment of palmitic acid, 2mM Sephin 1 (SigmaAldrich) was administered to the cells in increasing amounts for 2 hours. The cells were extracted from the six-well plates and lysed using pre-made 50 ul 1% RIPA buffer (Thermo Scientific). RIPA buffer located the protein at the membrane-bound, nuclear, and mitochondrial level for increased interest concentration. After collection, the mixture was centrifuged in 1.5 ml tubes at 4000 RPM for five minutes. The supernatant collected went through the process of sonication while kept on the ice twice. The protein concentration absorbance was measured using the NanoDrop application system to present the absorbance levels.

Sample preparation, electrophoretic separation of proteins using 12ul loading samples to run the SDS page gel for 1 hour at 150 volts, transfer of the separated proteins for two hours at 33 volts, and the milk blocking phase for 1.5 hours of the nonspecific sites was conducted before the nitrocellulose membranes were incubated with 10 ml primary antibodies against the specified kinases (Table 1). The process all occurred at room temperature before antibody incubation. After the transfer of proteins unto the membrane, the washing process of the nitrocellulose was conducted using a 1x TBST mixture of tris-buffered saline and polysorbate plus 0.1% Tween for 7-10 minutes in three intervals.
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<td>1:1000</td>
<td>Rabbit</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Santa Cruz</td>
<td>37</td>
<td>1:1000</td>
<td>Mouse</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Biotechnology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Primary Antibodies Used

After overnight treatment in the 4-degree cold room, the specified proteins were detected using the corresponding 10 ml secondary antibody and detected with a 200ul mixture of ECL Plus for chemiluminescent substrates signals (Table 2). Each kinase was tested around three times each to confirm activation of expression levels.

<table>
<thead>
<tr>
<th>Secondary Antibody</th>
<th>Company</th>
<th>Dilution Concentration</th>
<th>Animal Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Rabbit IgG</td>
<td>Cell Signaling</td>
<td>1:2000</td>
<td>Goat</td>
</tr>
<tr>
<td>Anti-Mouse IgG</td>
<td>Cell Signaling</td>
<td>1:2000</td>
<td>Goat</td>
</tr>
</tbody>
</table>

Table 2. Secondary Antibodies Used

iv. Statistical Analysis

All results were expressed as mean ± Standard Deviation. Data analysis was completed using the ImageJ software and Excel computational methods to generate the representative bar graphs shown in the results section. Each western blot plot was analyzed and compared to the GAPDH core control of the membrane.
3. Results

*Excessive accumulation of lipids led to cellular dysfunction.* After treating the Huh 7.5 cells, to verify the incorporation of the toxic acid into the cellular environment, immunofluorescence was conducted. The lipid accumulation is evident with the red stain, with minimal visible from the control (Figure 3). The nuclei, stained blue, were present in the merged imaging with DAPI. Disruption within the endoplasmic reticulum, mitochondria, and cellular lipid bilayer was affected by the excessively increasing saturated fatty acid accumulation.

*Integrated Stress Response activated the kinases and directly inhibited autophagy.* The presence of saturated fatty acids that led to the induction of the integrated stress response pathway resulted in the activation of all the specific kinases. PKR and ATF6 activated; however, minimal observations can be made. IRE1 alpha, P-EIF2 alpha, and GCN2 presented an increase in protein expression, while PERK, HRI, and LC3B presented a decrease in expression levels. LC3B decrease of the bands indicating the inhibition of autophagy due to the stressors. P62 increase due to the accumulation of fatty acid, further supporting the results of LC3B of autophagy prevention. MTORC1 presented high bands showing the inhibition of autophagy. The bar graph results are expressed compared to the central control, GAPDH, and quantifications assessed using ImageJ western blot analysis and excel.
Figure 3 Lipid Accumulation by Immunofluorescence Assay (A) Huh 7.5 cells stained with Oil Red O dye for the increasing doses of palmitate. The lipid accumulation was detected by 40X (magnification) fluorescence microscopy stained with DAPI.
The lipid bilayer response to integrated stress response impacts autophagy. Membrane fluidity PKR signal showed minimal changes to integrated stress response after protein analysis (Figure 4). The band intensity showed the kinase detected signaled cellular stressors; however, the balanced response to the stressors is not clear. The band result could be from the endoplasmic reticulum stress due to large quantities of unfolded proteins that activate the PKR kinase. The lysosomal membrane kinase MTORC1 experienced activation by the palmitic acid treatment before the hypothesized lipotoxicity resulted in a decrease (Figure 4). The fatty acid effect on the lysosomal kinase might have lead to inductive apoptosis, causing the decline in activation. The progressively increasing GCN2 demonstrates that its activation in response to integrated stress induction furthers inhibits translation, proving autophagy inhibition (Figure 4). If the MTORC1 kinase presented activating bands, it could have added proof of autophagy since it stimulates protein translation. The inhibition of protein synthesis results from the integrated stress response; therefore, the results reveal the impact of the response pathway on autophagy.

Figure 4 (A) Immunoblot analysis for PKR, MTORC1, and GCN2 per increasing dosage (B-D) Shows the levels of expression induced by integrated stress response on the protein kinases.
The endoplasmic reticulum is induced from the induction of ISR. The endoplasmic reticulum PERK and ATF6 alpha decrease after the 24-hour treatment of palmitic acid to reduce ER stress (Figure 5). The inhibition of the ER stress kinase and expected induction of protein synthesis differ from the analysis of the lysosomal and protein kinases, possibly due to the extended capabilities of the ER. ISR imposing effect on the PERK and ATF6 kinases show a possible inductive effect in autophagy. If the cells were treated for 48 hours and 72 hours and assessed by western blot analysis, the effect of the induced ISR would show clarity and is expected to lead to the inhibition of autophagy due to lipotoxicity. IRE1 alpha activated in expected response to the unfolded proteins for repair (Figure 5). The effects of IRE1 alpha in fatty acid metabolism and UPR signaling is an area that is still being studied by researchers. The researchers found that the activation of IRE1 exhibited chemo-tolerance in liver cancer cells, including possible mechanistic approaches to the delivery of therapeutics or fatty liver disease biomarkers.

Figure 5 (A) Displays analysis of increasing 10mM Palmitate for IRE1 alpha, PERK, and ATF6 alpha. (B-D) Show the quantification expression induced by integrated stress.
**Integrated Stress Response induces Mitochondrial stress in liver cells.** The heme-regulated inhibitor, HRI, decreased significantly due to integrated stress response before a spike increase occurred (Figure 6). The signaling response of the mitochondrial kinase provided unidentified reasoning for the presented band. A future direction for the mitochondrial stress kinases will be to assess palmitate-induced ISR on OMA1, Metalloendopeptidase, kinase, a human mitochondrial precursor.

**Figure 6 (A)** Exhibits the western blot analysis levels to specific organelle kinase after 24-hour treatment or increasing 10mM Palmitate and protein concentration assessment doses. **(B)** The proliferating interpretation of HRI quantification to match analysis in part A.

**Palmitate toxicity effect on P-EIF2 alpha regulation.** P-EIF2 alpha phosphorylation increased in response to the ISR pathway activation (Figure 7). The sustained activation of the kinase combats integrated stress response since P-EIF2 alpha has translational control through its structural conformational changes within the cell. The data demonstrate that the fatty acid treatment causes inhibition of autophagy, although P-EIF2 alpha was activated in expected support for the disturbed organelles. The increased proliferation of P-EIF2 alpha was hypothesized to induce autophagy; however, in this study, autophagy was inhibited. The significant EIF2 kinases, PKR, HRI, GCN2, and PERK, from other studies, all elicited diverse responses to influence P-EIF2 alpha activation; However, the reaction to the ISR activation occurred in a conducive manner for each kinase regulatory capabilities. The integrated stress response is connected to many diseases like liver disease and serves as a therapeutic target. It regulates the activation of the P-EIF2 alpha and cell kinases. During ISR, protein synthesis is inhibited by the phosphorylation of P-EIF2 alpha. The future direction for this project is to use the results achieved for developing immune response mechanisms or biomarkers dependent on P-EIF2 alpha to reduce hepatic inflammation and progressively resolve liver problems.
**Palmitate-induced Integrated Stress Response inhibited autophagy.** Palmitic acid inhibited autophagy through the LC3B marker, supporting the overall expectation of inhibition (Figure 7). The LC3B protein was a reliable biomarker for breast cancer patients (41). Inhibition of autophagy can serve as a new pathway of improving chemotherapy treatment in liver cancer patients (Lefort). ISR induction from saturated fatty acids, led to the inhibition of autophagic influx for cellular repair. The multifunctional protein, P62, elicited a strong expression supporting the results of LC3B concerning the modulation of autophagy. Understanding how the input of fatty acids contributes to integrated stress response in cellular stressed liver cells was assessed; the incidence of autophagy and vividly apoptosis is clear from the study. In support of the hypothesis, we showed that induced integrated stress response by saturated fatty acids did activate autophagy in response to cellular stressors. Our data supported the notion that saturated fatty acids inhibit autophagy through the persistence of P-EIF2 alpha.

**Figure 7 (A)** Presents the blot analysis levels due to integrated stress response in autophagy marker LC3B and activator P-EIF2 alpha compared to GAPDH control. **(B)** The expression of LC3B alpha by ImageJ analysis. **(C)** Imaging analysis of P-EIF2 alpha of western blot results. **(D)** Autophagy receptor, P62 expression increased.
**Sephin 1 incubation led to inhibition of P-EIF2 alpha.** Sephin 1, 2 hours administered drug treatment in 24-hour Palmitic acid-treated cells produced supportive results concerning the fate of autophagy. The sephin 1 treated portion elicited inhibitory effects in P-EIF2 and activation of autophagy in the LC3B kinase (Figure 8).

![Figure 8](image_url)

**Figure 8** (A) Presents the blot analysis levels of Sephin 1 contribution to integrated stress response in P-EIF2 alpha expression and LC3B. (B) The expression of the vital kinases was assessed by ImageJ analysis. The drug treatment followed control, palmitate 0.05uM, Palmitate 0.1uM, Palmitate 0.25uM, Palmitate 0.25um + Sephin 0.25uM, Palmitate 0.25uM + Sephin 0.5uM, and Palmitate 0.25uM + Sephin 0.75uM.
Figure 9 Overall Immunoblot Expression of Palmitate (A) Shows the expression levels to each organelle kinase compared to GAPDH control.
4. Discussion

Background

Chronic fatty liver, associated with various liver abnormalities, will lead to liver carcinoma and eventually cause death. The stages begin with simple fatty liver, before steatohepatitis due to inflammation from the excess fat, then fibrosis scarring, and eventually, cirrhosis, also known as widespread scarring. The molecular mechanism that drives the progression of liver carcinoma from fatty liver exposure is a phenomenon that researchers continue to investigate to find suitable and effective treatments. The overall rate of nonalcoholic fatty liver disease in African countries varies greatly. The estimated percentage of Africans dealing with fatty liver disease ranges from 50-80%, with the rising prevalence of obesity, diabetes, and various liver suppressive illnesses (10). The modern diet in the United States and African countries is high in saturated fats, leading to overcompensation of organelles to digest and transport stored fat to the liver. At adequate amounts, the fat storage is used from energy during the fasting phases; however, a gradual accumulation of the fat will lead to disease.

Fatty liver disease diagnosis frequently occurs in obese patients and individuals that follow a high fat, high sugar diet. A study conducted by Garcia-Berumen et al., 2019, discovered that a diet of high fructose levels inhibits the metabolism of fats in the liver, thereby damaging cellular mitochondria oxidation (41-42). The buildup progression of fatty liver disease due to fat accumulation to advanced liver disease occurs within ten years. Whether simple fatty liver formulates into a more severe medical condition independently, without stressors, is a vital angle that should be further researched. Likewise, the irreversibility of fatty liver, before it progresses to advanced liver abnormalities like cirrhosis, is an additional angle to assess therapeutics' production.

The mechanism that palmitate tasks to infiltrate the cellular membrane before reaching the cellular organelles is not well understood. It is known that the toxic acid enters the cell by breaking through the bilayer and attacks the cells; there is a high chance that the current cancerous cells within an organelle will experience a proliferation (35). The treatment of palmitate is proven, through multiple studies, to induce lipotoxicity that results in organelle dysfunction and cell death. Nonetheless, further understanding how this fatty acid affects stressed cells and how that could impact autophagy was the aim of this study and the
focus of many scientists. A prolonged diet strictly filled with fats and sugar will lead to elevated stressors for a significant amount of time in human cells. An individual following an unhealthy diet with a proliferating liver abnormality should have cellular kinase irregularities if PERK, IRE1 alpha, PKR, HRI, ATF6 alpha, GCN2, MTORC1, LC3B, and P-EIF2 alpha were assessed from the liver hepatocytes. The lipid overload caused by the acids is related to the pathology and progression of obesity and type 2 diabetes leading to cytotoxic signaling and cell damage (36-37). The integrated stress response to this signaling adds an interesting take on the role of palmitic acid on the initiation or inhibition of autophagy and lysosomal dysfunction. The pathway that the saturated fat entails modulating autophagy was thought to be through the P-EIF2 alpha pathway.

P-EIF2 alpha is the phosphorylation of the eukaryotic initiation factor 2 that down-regulates proteins in stressful conditions. It is a facilitator for cells to lead towards adaptations with the stressful environment (38). The kinase works to shift cells from an apoptotic condition to a cytoprotective state in response to several stressors, such as glucose deficiency (39). GCN2, PKR, and the endoplasmic reticulum kinases, IRE1 alpha, ATF6 alpha, and PERK all play a role in how P-EIF2 alpha conducts cytoprotective mechanisms. A few kinases are apoptotic inducers, such as GCN2, while the others work along with the P-EIF2 kinase for cell survival (39). The phosphorylation P-EIF2 alpha kinase interdependency on autophagy is a vital connection to determine the extent to which the kinase will input the modulation of autophagy. P-EIF2 alpha and the cellular organelle kinases are interdependent, possibly resulting in the P-EIF2 alpha pathway's experiencing a stressful workload. Endoplasmic reticulum stress signaling leads to increased protein misfolding to transduce IRE1 alpha, PERK, and ATF6 activation. The activated kinases all phosphorylate P-EIF2 alpha to promote protein translation to relieve the stressors. The phosphorylation of the P-EIF2 alpha kinase also enhances the translation of the ER stress kinase to promote translocation to nearby organelles to drive chaperone expression, chaperone translation and results in the slicing of mRNA (38-40). In the mitochondria, HRI, membrane PKR, and protein kinase GCN2 are activated by the integrated stress response trigger intense P-EIF2 alpha phosphorylation that can inhibit tumor growth at a non-toxic dose (40). At toxic doses, HRI that prosurvival outcome and adaptation to the stress response of P-EIF2 alpha is a questionable process that can further be researched.
Lysosomal MTORC1 impact on the downstream pathways to control survival and homeostasis can inhibit the phosphorylation of P-EIF2 alpha due to its suppressive capabilities if it is not stressed. The connective effects of P-EIF2 alpha to the organelle kinases that should potentially be activated are questionable. Deactivation of a particular kinase could result in inhibition or induction of P-EIF2 alpha; however, the effects of the other kinases on it could disturb its activation of autophagy. To get the crosstalk between the kinases and EIF2 alpha signaling pathways to be the same, this would be in a theoretically homeostatic environment. The dephosphorylation effects of P-EIF2 alpha lead to an inhibition of the kinase when treated with Sephin 1. With the drug treatment, autophagy activated as P-EIF2 alpha expression decreased. The reversal role in the kinases elicits evidence that integrated stress response can be combatted through therapeutic measures (Figure 10).

**Figure 10 Pharmacological approach of Sephin 1 on components targeting ISR**

In this present study, examining the direct effects of palmitate on integrated stress response and the modulation of autophagy through the P-EIF2 alpha pathway was conducted. It has been demonstrated that saturated fatty acid proliferation in the cellular environment will cause cellular disturbances, endoplasmic reticular stress, mitochondrial stress, and inhibition of protein synthesis. The kinases of each organelle were carefully chosen to represent the significance played during stress activation (Figure 2). The
unrealistic expectation of all the kinases increasing due to the induction of integrated stress response would not elicit evidence-based results and further show which kinase was activated or inhibited (Figure 9). Knowing the different reactions of each kinase can lead to further study of the role it plays in ISR therapeutics. Taken together, the results establish the critical need to develop a therapeutic approach to dealing with fatty liver disease progression.

**Study Limitations**

A limitation of the study concerned the dosage of palmitate that could have been administered at lower treatment times, such as six hours and twelve hours. Likewise, using non-stressed hepatocytes would have yielded the expected results; however, to further research to develop treatments, the use of stressed tumorigenic cells was necessary.

**Conclusion**

When exposed to environmental or internal stress, Eukaryotic cells activate ISR to restore homeostasis and promote cell survival. The establishment of the exact pathway that responded positively to integrated stress response and led to an activation of autophagy was the theoretical pathway to assess further. According to the results of this research, the ER pathway upheld the most promising results, including the P-EIF2 alpha, an integral member of the ER kinases. The importance of studying the pathways that our cells take to alleviate cellular stress and fat accumulation was assessed for this research project.

**Future Directions**

If permitted for additional research time, the P-EIF2 alpha pathway would be assessed further to better understand the kinase's interdependency with autophagy. The next step would be to destabilize and also induce the phosphorylation of P-EIF2 alpha with drug treatments. ISRIB, a potent and selective inhibitor of the integrated stress response mechanism that reduces adaptation to endoplasmic reticulum stress, would be used as the P-EIF2 alpha inhibitor (Figure 10). The counteractive effects of P-EIF2 alpha on autophagy by Sephin 1, elongator of the integrated stress response and an inhibitor of P-EIF2 alpha dephosphorylation, served as a strong drug treatment choice. After treating palmitic acid for 24 hours in
two six-well plates, each drug will be treated to increase doses for the recommended treatment time.
Western blot analysis of P-EIF2 alpha, LC3B, P62, PERK, and GAPDH would be the suitable kinases for
the representative drugs. After results are consistent with the inhibition or induction of autophagy based on
the impact of the drug on the fatty acid cellular environment, immunostaining for each treatment dose could
be completed to match the western blot analysis. To understand how P-EIF2 alpha inhibition or activation
on autophagy could provide protective strategies for integrated stress response to provide a promising
therapeutic approach for progressive fatty liver disease.
LIST OF REFERENCES


ABBREVIATIONS

ISR also named Integrated Stress Response

PKR, also named Protein Kinase R

IRE1 alpha, also named Serine/Threonine-protein kinase/endoribonuclease

PERK, also named protein endoplasmic reticulum kinase

ATF6 also named Activating Transcription Factor 2 alpha

P-EIF2 alpha also named phosphorylated eukaryotic initiation factor 2 alpha

HRI also named Heme Regulated Inhibitor 1

MTORC1 also named mammalian target of Rapamycin complex 1

GCN2 also named general control nondepressible 2 protein kinase

LC3B, also named light chain 3B

P62, also named Sequestosome 1 – SQSTM1 gene, encodes

GAPDH, also named Glyceraldehyde 3-phosphate dehydrogenase

ER, also named Endoplasmic Reticulum
BIOGRAPHY

Ms. Ogunyinka is an Ekiti Yoruba-Nigerian first-born daughter of three, born in Mannheim, Germany, to Dr. (Pastor) Ebenezer and Mrs. (Deaconess) Beatrice Ogunyinka. She completed her undergraduate degree in Kinesiology, Pre-Dentistry from the great Louisiana State University, Baton Rouge, LA, in May of 2019. Still focusing on her goal of becoming a dentist, Ms. Ogunyinka decided to complete a master's degree in Molecular and Cellular Pathobiology from Tulane University School of Medicine, New Orleans, LA, in May 2021. She built intense research and analytical skills through the mentorship provided in her lab and within the Pathology department. Ms. Ogunyinka will be attending Tufts University School of Dental Medicine beginning July 2021.