The Effects of Chronic Insulin Exposure on Triglyceride Transfer Protein in Adipocytes

ABSTRACT

According to the WHO and CDC, 40% of all US adults are obese and more than 100 million US adults are now living with diabetes or prediabetes, respectively. The adipose is a multi-depot organ that regulates glucose homeostasis. Since microsomal triglyceride transfer protein (MTP) is present in both white and brown adipocytes and is a lipid transfer protein, 3T3-L1 adipocytes were used to determine the relationship between insulin resistant adipocytes and MTP. After a cell culture was done, the 3T3-L1 cells were differentiated into adipocytes. Subsequently, the adipocytes went under chronic insulin exposure (CIE) and were given 500 pM of insulin for 72 hours to form increased lipid droplet size and accumulation, which are common risk factors for insulin resistance. An MTP Activity Assay and an RT-PCR were done to identify MTP activity and MTP expression at an RNA level, respectively. The results indicated a decrease in MTP activity due to a decrease in MTP expression under chronic insulin conditions. It is rational to suggest MTP expression and activity play a critical role in adipocyte insulin resistance. Further research is required to determine the mechanism by which insulin resistance regulates the expression of MTP. The experimental results suggest that MTP can be a potential therapeutic target for insulin resistance.

Introduction

As reported by the World Health Organization (WHO, 2018), nearly 40% of the US adult population are obese. Obesity is characterized by an excess accumulation in fat mass of adipose tissue. Doctors use body mass index (BMI) as a tool to assess people's weight and determine if one is obese. A BMI between 25 and 29.9 indicates excess weight, and a BMI of 30 or above indicates obesity. Obesity can be caused by a sedentary lifestyle, not sleeping enough, medications, and consuming too many calories. It may increase the risk of developing arthritis, cancer, and metabolic syndrome, which is a collection of issues, including cardiovascular diseases and type 2 diabetes (Brazier and Marengo, 2018).

With obesity on the rise, bariatric surgery has become a popular solution. The most common of the bariatric surgery procedures are gastric bypass, and sleeve gastrectomy. Bariatric surgical procedures can cause weight loss by restricting the amount of food the stomach can hold. Many of the procedures can cause long-term vitamin deficiency, slow weight loss, dilation of the esophagus, and risk for mortality (ASMBS, 2019). Since 13.7 million children face obesity (CDC, 2017), pediatricians are beginning to suggest bariatric surgery as a short-term solution for drastic weight gain. Though bariatric surgery reduces both the size of the individual adipose depots and adipocytes, it is unclear if surgery is a long-term solution (Schmidt *et al*., 2016).

Fat cells, or adipocytes, are major energy storage sites in the body. There are three general classes of adipocytes, including white, brown, and brite adipocytes. Brown adipocytes store energy in small lipid droplets to use as fuel in thermogenesis, body heat. The white adipocytes store energy as a single large lipid droplet. They have very important endocrine functions. The brite adipocytes are formed through the browning of white adipocytes or vice versa, depending on temperature and diet. Active brown adipocytes have interesting metabolic programs. They can consume large amounts of nutrients, including glucose, lipids, and amino acids, simultaneously, while engaging in both anabolic and catabolic metabolism. The white adipocytes are the most abundant adipocytes in humans. They signal feeding behavior and metabolic homeostasis to the brain. Though they have an ability to expand and store energy, white adipocytes have a tipping point in which their beneficial functions fail-obesity (Adipocytes, 2016). This may promote the onset of type 2 diabetes.

According to the CDC (2017), 84.1 million US adults have prediabetes or type 2 diabetes. Type 2 diabetes results from the body's ineffective use of the hormone insulin, secreted by the pancreas, resulting in elevated levels of glucose in the blood. Type 2 diabetes is mainly caused by obesity, or an excess of adipocytes in the body. It may cause cardiovascular diseases, foot damage, retinopathy, neuropathy, and Alzheimer's disease (CDC). This pandemic has become a real threat to the health of the nation.

Glucose homeostasis is the balance of insulin to maintain blood glucose levels, but it is not maintained under diabetic conditions (Sturm, 2019). Insulin lowers blood glucose by increasing glucose

uptake in adipose tissue. Glucose homeostasis is maintained by three major parts of the body: liver, adipose tissue, and skeletal muscle. Slight impairment in adipose tissue function will have colossal effects on glucose homeostasis, leading to the development of insulin resistance (IR) (Cignarelli *et al.,* 2019).

Insulin resistance is defined as a condition in which cells fail to respond to a normal concentration of insulin. This condition has been considered a major risk factor for type 2 diabetes (Shanik *et al.,* 2008). Among the three organs that help maintain glucose homeostasis, the function of microsomal triglyceride transfer protein (MTP), an important lipid transfer protein, is well studied in the liver. Studies have depicted that insulin negatively regulates MTP expression in hepatic cells (Higuchi *et al*., 2011). As a result, there is a buildup of triglycerides in the cells, which can cause obesity. Two independent groups Bakillah and Hussain (2016) and Swift *et al*. (2017) have shown the expression of MTP in white and brown adipocytes.

Microsomal triglyceride transfer protein (MTP) is an essential tool for the synthesis of apolipoprotein-B in the liver and intestine (Dai and Hussain, 2012). MTP also facilitates in the transfer of triglycerides, cholesterols, and esters between membrane vesicles (Dhote *et al.,* 2011). Since MTP is the chaperone for the transfer of triglycerides, it might have an essential role in adipocyte functioning and disease pathology as MTP stores triglycerides in the form of lipid droplets.

MTP is essential for Apo-B synthesis and secretion of lipoproteins, its role is mainly characterized in the liver and intestine. It was later discovered that MTP has eleven domains and facilitates the transfer of phospholipids and sphingomyelin. Recently, Bakillah *et al.* (2016) showed that MTP is also expressed in adipose tissue. Adipose tissue specific MTP knockout mice are lean and resistant to high fat diet induced obesity. The mechanisms behind the lean phenotype of the mice was not clear. Another study conducted by Swift *et al.* (2017) validated these findings as it showed that MTP expression increases during differentiation, but the inhibition of MTP had no effect on 3T3-L1 preadipocytes. Although these results show MTP expression in adipose tissue, it fails to explain the function of MTP in adipose tissue. Therefore, the goal of this research project is to identify the role of chronic insulin treatment on MTP activity and expression in adipocytes.

Materials and Methods

3T3-L1 Cell Culture

Media was made using 500 mL DMEM, 50mL FBS, and 5 mL of L-glutamine; sterilization of the media was done through vacuum filtration. One 3T3-L1 cell vial (ATCC) was taken off ice and 1mL of media was added to the 3T3-L1 vial and resuspended until homogenized. This mixture was pipetted into a 15 mL Falcon tube and centrifuged at 300 g for five minutes. Once centrifuged, the mixture was taken out, and resuspended. All the content was removed from the Falcon tube and placed into 2 labeled flasks. 12 mL of media was then added to the flasks as well.

After 48 hours, the cells were split by removing all the media from the flask and adding 1-2 mL of Trypsin-EDTA (10x). The flask was incubated for 30 seconds. To ensure that all the 3T3-L1 cells were mobile, the flask was tapped and observed under the microscope. To inactivate the trypsin, 10 mL of media was added to the flask. The media/cell mixture was then transferred to a 50 mL tube. The media/cell mixture was centrifuged at 1200 rpm for 5 minutes. The media above the pellet was removed and 10 mL of media was added to the pellet and resuspended. 1mL of the resuspended cells and 9 mL of media were added to the culture flask, creating a 1 to 10 dilution. The flask was left in the 4℃ incubator until the cells were 80% confluent.

Once 80% confluent, the media in the flask was removed and 2 mL of trypsin was added. After waiting 60 seconds, the flask was tapped to ensure that all the cells were mobile. 8 mL of media was added to the flask and pipetted up and down. The media/cell mixture was added to a 15 mL Falcon tube and centrifuged at 1200 RPM for 5 minutes. The top media was removed, and the pellet was kept. 1 mL of media was added to the tube and resuspended. Once 24 mL of media was aliquot into a 50 mL Falcon tube, the media/cell mixture was added into it. 2 mL of this mixture was then added to each well. After 72 hours, the media was removed and re-added.

3T3-L1 Cell Differentiation

After the 72-hour growth period, the 3T3-L1 cells became 100% confluent and ready for differentiation. The media was aspirated and 2 mL of the first differentiation cocktail was administered. The cocktail included 250 µL of IBMX (500 µM), 125 uL of Insulin (5µg/mL), 25 µL of Dexamethasone (250 nM), 24,600 µL of media, and 0.5 µL of Rosiglitazone. After another 72 hours, the media will be aspirated and 2 mL of the second differentiation cocktail was administered. This cocktail included 125 µL of insulin and 24,875 µL of media. 48 hours later, the media was aspirated, and 2 mL of media were added to each well.

Oil Red-O Staining

An Oil Red-O stock was created by adding 0.35 g of Oil Red-O dye to 100 mL isopropanol (100%). Using the stock, a working solution was made by adding 4 mL of deionized water to 6 mL of the stock solution. The media that was added to the 3T3-L1 adipocytes was removed, and the cells were washed in a 6 well-plate with PBS (1X) three times. Once the cells were washed, 1 mL of the working solution was added to each well and incubated at 37℃ for 15 minutes. Once incubated, the cells were washed three times with PBS (1X), but unlike before, the cells were not aspirated after the third wash. Images of the stained cells were then taken with a confocal microscope (Nikon Eclipse TE300) after 24 hours 48 hours, and 72 hours. 1 ml of isopropanol was added to each well, the plate was swirled, and the plate was incubated for 5 minutes (Kraus et al. 2016). After incubation, 100 uL of the solution was added

to each well of the 96 well plate and read and analyzed at 490 nm using a spectrophotometer (Perkin Elmer VICTOR 3). The absorbance was read to quantify the lipid droplet content of each well.

MTP Activity Assay

Proteinase K buffer (Buffer K), containing EDTA, NaCl, and Tris-Cl with Protease Inhibitor Cocktail (PIC) was added to the control and CIE induced adipocytes. The cells were scraped and divided into eppendorf tubes and placed into ice. The cells were then agitated by flicking so the cells would rupture. Then the cells were centrifuged for 10 minutes at 1200 RPM. The supernatant was removed and placed into eppendorf tubes. A protein standard containing BSA (50 mg/mL) and water was added to two columns of the 96 well plate. 10 µL of the CIE and control adipocyte supernatants were then placed into another two columns of a 96 well plate. 100 µL of Pierce BCA Protein Assay Reagent B and Copper Sulfate mixture were added to all the wells. A protein estimation using the Assay Start Wizard was then done at 562 nanometers. Once the protein concentration for all samples were unified, an MTP Activity Assay was done in triplicate. 5 mL of triglyceride vesicles were added to each well of a new plate. 95 µL of the sample, control, Buffer K, and Isopropanol were also added to the plate, respectively. The positive control was the chronic insulin exposed (CIE) adipocytes while the negative control was the adipocytes. The plate was tapped and read at 0 minutes, 5 minutes, 10 minutes, 15 minutes, 20 minutes, 45 minutes, and 60 minutes, using the Perkin Elmer Enspire Multimode Plate Reader. The percent transfer was calculated by subtracting the Buffer K absorbance from the CIE absorbance and dividing that by the Buffer K absorbance subtracted by the Isopropanol absorbance. This value was then multiplied by 100. The percent transfer has a direct relationship with MTP activity.

qRT-PCR

Total RNA was isolated from 3T3-L1 adipocytes by adding 1 mL of TRIzol reagent that contains properties, such as phenol, guanidine isothiocyanate, and red dye to the 6-well plate. With a pipette, the cells were dislodged from the 6-well plate and were placed into epindrophs. 250 µL of Trichloromethane (chloroform) were placed into each eppendorf tube. The eppendorf tubes were then centrifuged and the supernatant was removed, leaving solely the RNA in the eppendorf tube. 1.4 mL of 75% Ethanol was added to each RNA filled eppendorf tube to prevent contamination. Using the NanoDrop™ 2000/2000c Spectrophotometer, the RNA concentration of the CIE and control adipocytes was determined. The isolated RNA was then converted into cDNA using the high capacity cDNA reverse transcription kit. 10x RT buffer, 100 mM deoxyribonucleotide triphosphate (dNTPs), RT primer, RT enzyme, and Ultrapure water were used to go forth with the reaction. This again was used for the quantitative real time PCR analysis on LightCycler 480, using SYBR Green master mix. The statistical analysis of the qt-PCR was done using relative changes in gene expression as compared to the reference gene, 18S rRNA.

Data Analysis

To analyze and determine statistical significance ($p<0.05$) of the results of the MTP Activity Assay and the Real Time PCR, Student's *t* tests were done in GraphPad Prism.

Results

3T3-L1 adipocyte show increased lipid droplet accumulation

To determine the success of the 3T3-L1 differentiation, the differentiated cells were stained with Oil Red-O dye and observed under a confocal microscope (Nikon Eclipse TE300). Unlike the fibroblasts, the stained and unstained adipocytes formed circular lipid droplets (Figures 1 and 2). Compared to 3T3-L1 preadipocytes, both stained (Figure 1) and unstained (Figure 2) 3T3-L1 adipocytes showed increased lipid droplet accumulation.

CIE induced adipocytes showed increased lipid droplet accumulation and size in comparison to the control

After 72 hours of inducing chronic insulin treatment, the CIE and control adipocytes were stained with Oil Red-O dye and observed under a confocal microscope (Nikon Eclipse TE300). Unlike the control adipocytes, the CIE adipocytes had larger lipid droplets and had a greater amount of them, specifically at the 72-hour mark (Figure 3). To quantify this data, an absorbance of the Oil Red-O dye was done at 490 nM using a spectrophotometer (Perkin Elmer VICTOR 30). Unlike the first 48 hours, at 72 hours there was a significant ($p< 0.01$) difference in absorbance between the control adipocytes and the CIE (Figure 4).

MTP activity was less in CIE adipocytes in comparison to control adipocytes

An MTP Activity assay supported the significant ($p< 0.001$) difference of MTP activity between the control adipocytes and the chronic insulin exposed (CIE) adipocytes at the 30-minute mark (Figure 5). At the 45-minute mark, the control adipocytes had a significantly ($p< 0.01$) greater amount of MTP activity in comparison to the CIE adipocytes (Figure 6).

The expression of MTP was greater in the control adipocytes in comparison to the CIE adipocytes

With a qRT-PCR, the MTP expression of CIE and control adipocytes were determined at an RNA level. The relative mRNA expression is directly related to the MTP expression. MTP expression in CIE adipocytes was significantly ($p< 0.01$) lower than the expression in control adipocytes (Figure 7).

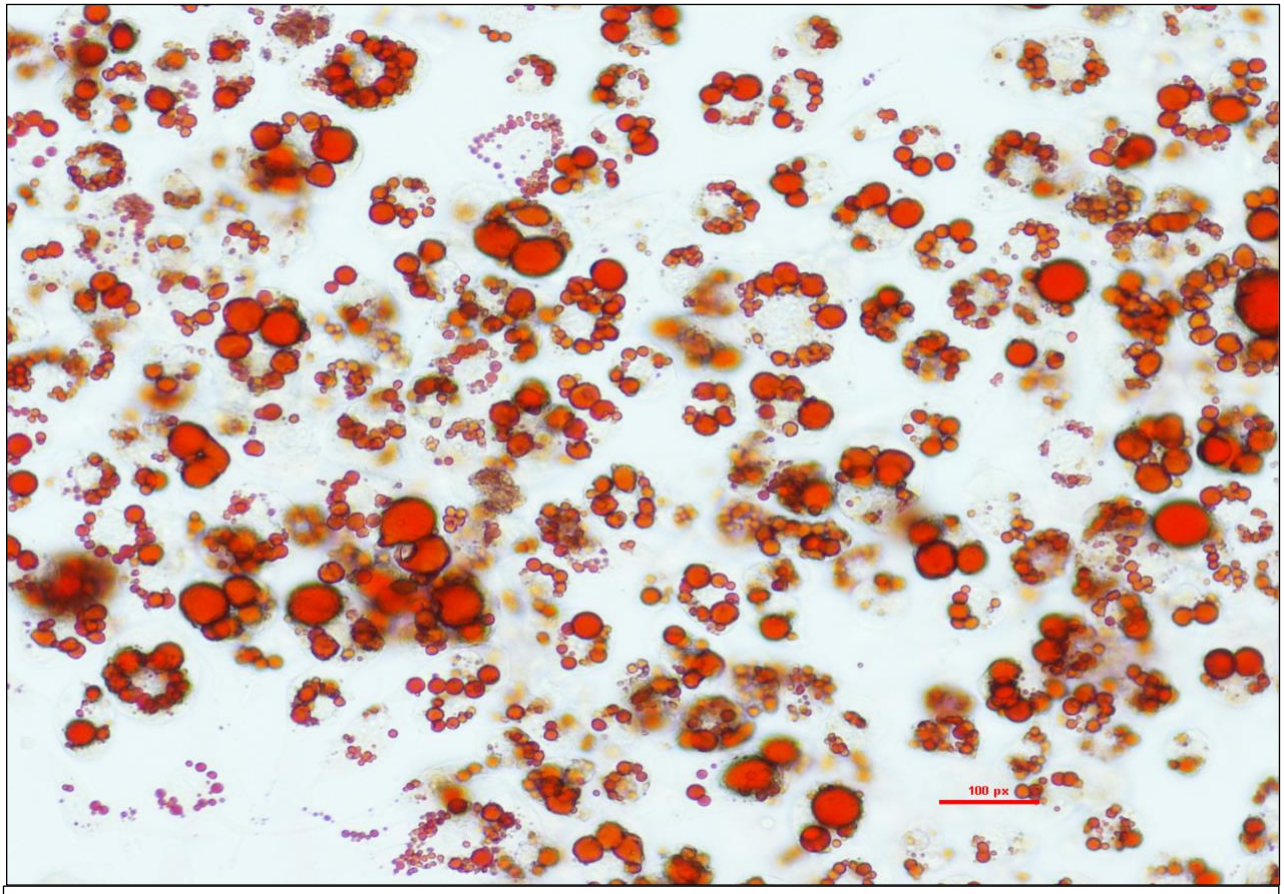

Figure 1 Image of Oil Red-O stained 3T3-L1 adipocytes (100 px) using a confocal microscope (Nikon Eclipse TE300). A great accumulation of circular lipid droplets formed depicting the success of the differentiation (created by student researcher).

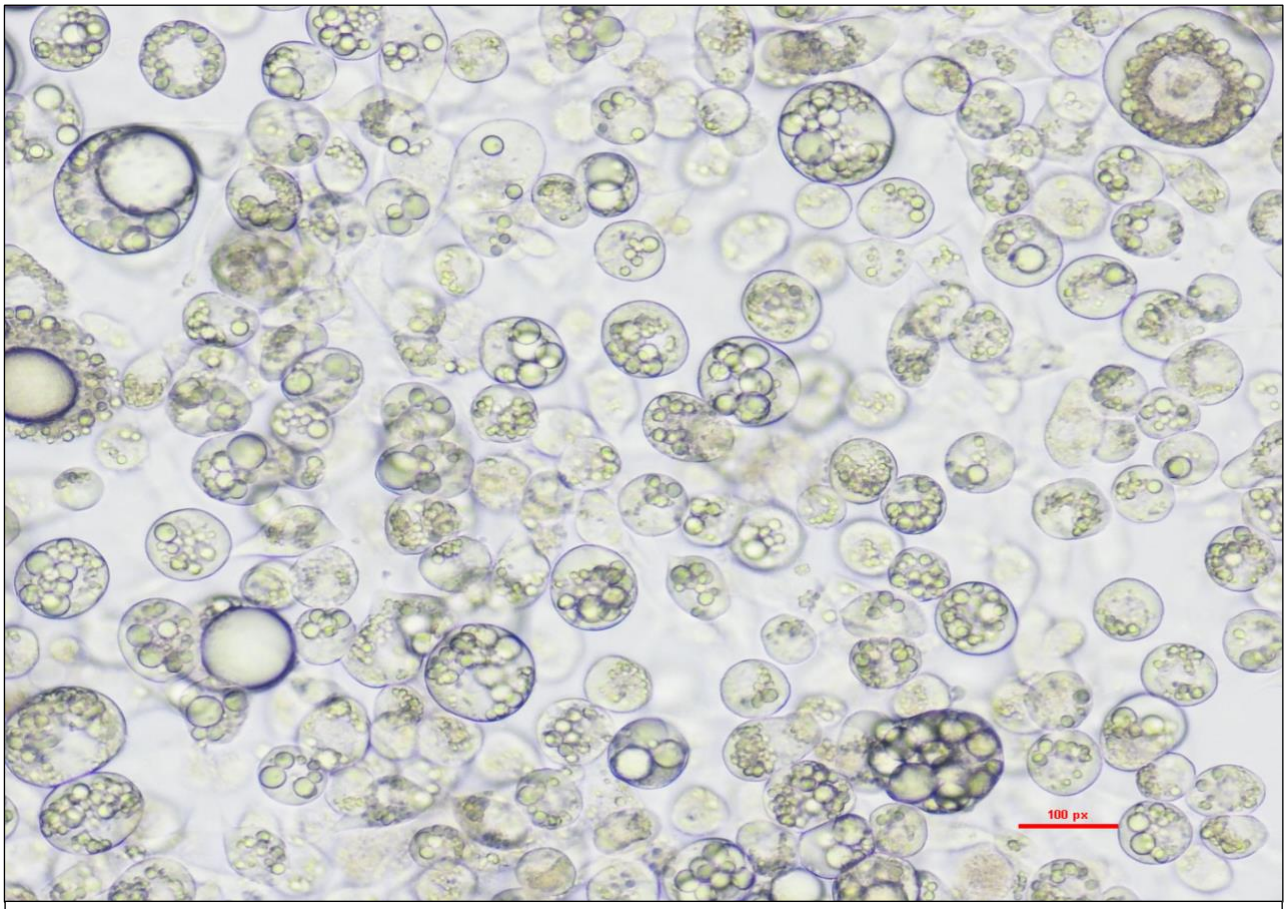

Figure 2 Image of unstained 3T3-L1 adipocytes (100 px)using a confocal microscope (Nikon Eclipse TE300). A great accumulation of large, circular lipid droplets formed depicting a successful adipogenesis (created by student researcher).

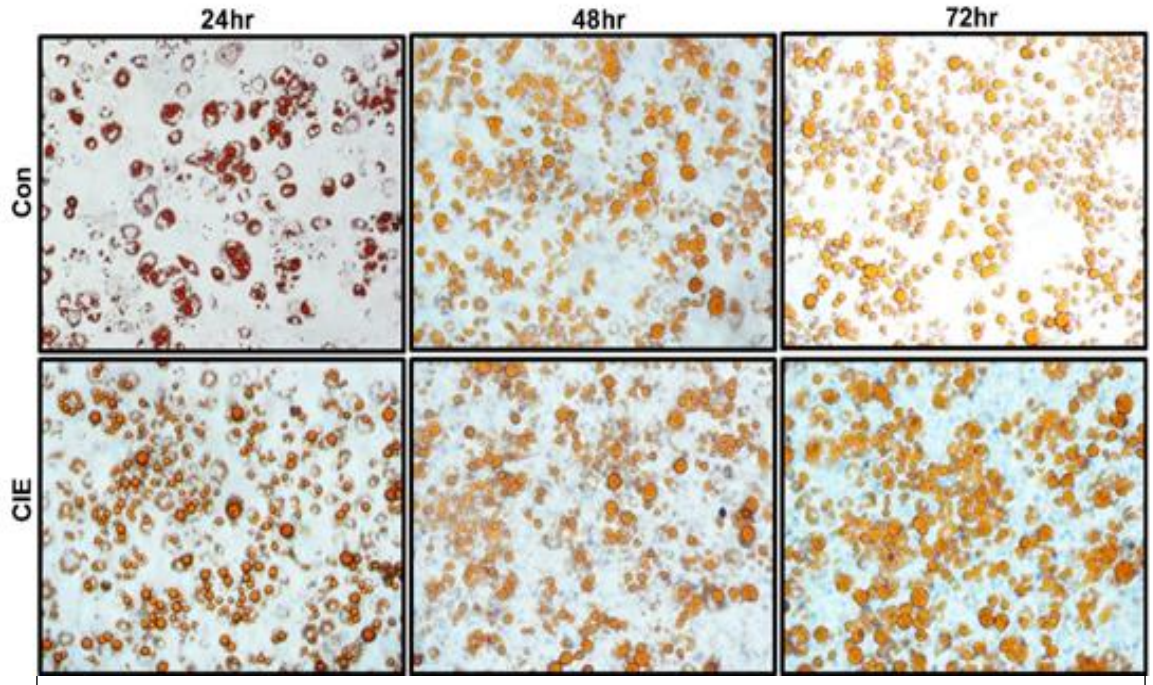

Figure 3: Three-day side by side image of the Oil Red-O stained control adipocytes vs. the CIE adipocytes under a confocal microscope (Nikon Eclipse TE300). After 72 hours, the CIE induced adipocytes had larger lipid droplets and a greater amount of them in comparison to the control adipocytes (created by student researcher).

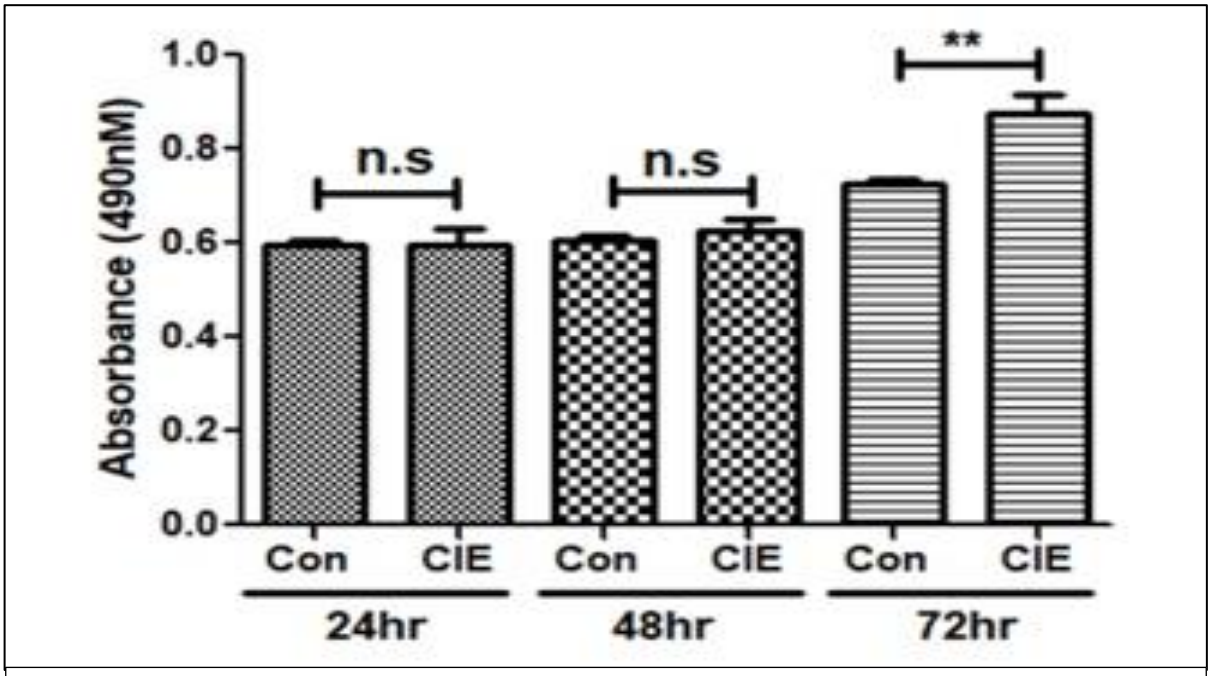

Figure 4: The Oil Red-O staining was quantified using the spectrophotometer (Perkin Elmer VICTOR 3) at an absorbance of 490 nM. There was significant ($p < 0.01$) difference at 72 hours between the absorbances, directly relating to the lipid droplet accumulation (created by student researcher).

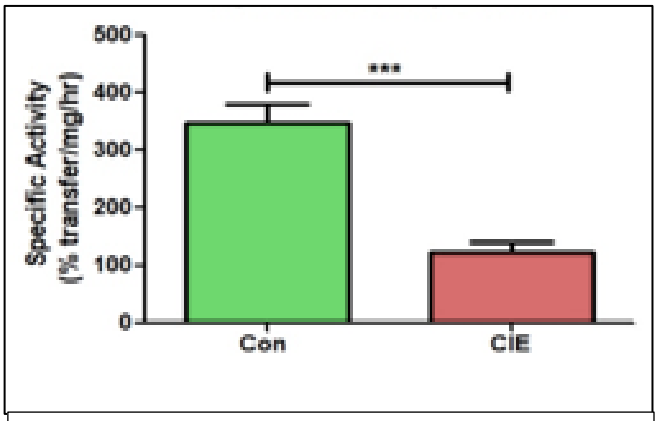

Figure 5: At the 30 minute mark, the percent transfer of the control adipocytes was significantly ($p < 0.001$) greater in the control adipocytes in comparison to the CIE adipocytes, indicating greater MTP activity (created by student researcher).

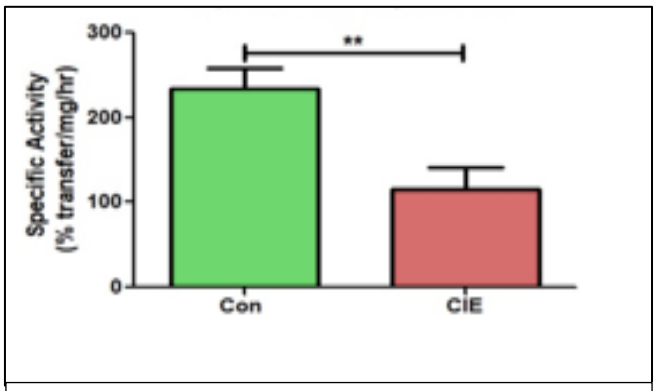

Figure 6: At the 45 minute mark, the percent transfer of the control adipocytes was significantly ($p < 0.01$) greater than the CIE adipocytes, indicating more MTP activity in the control (created by student researcher).

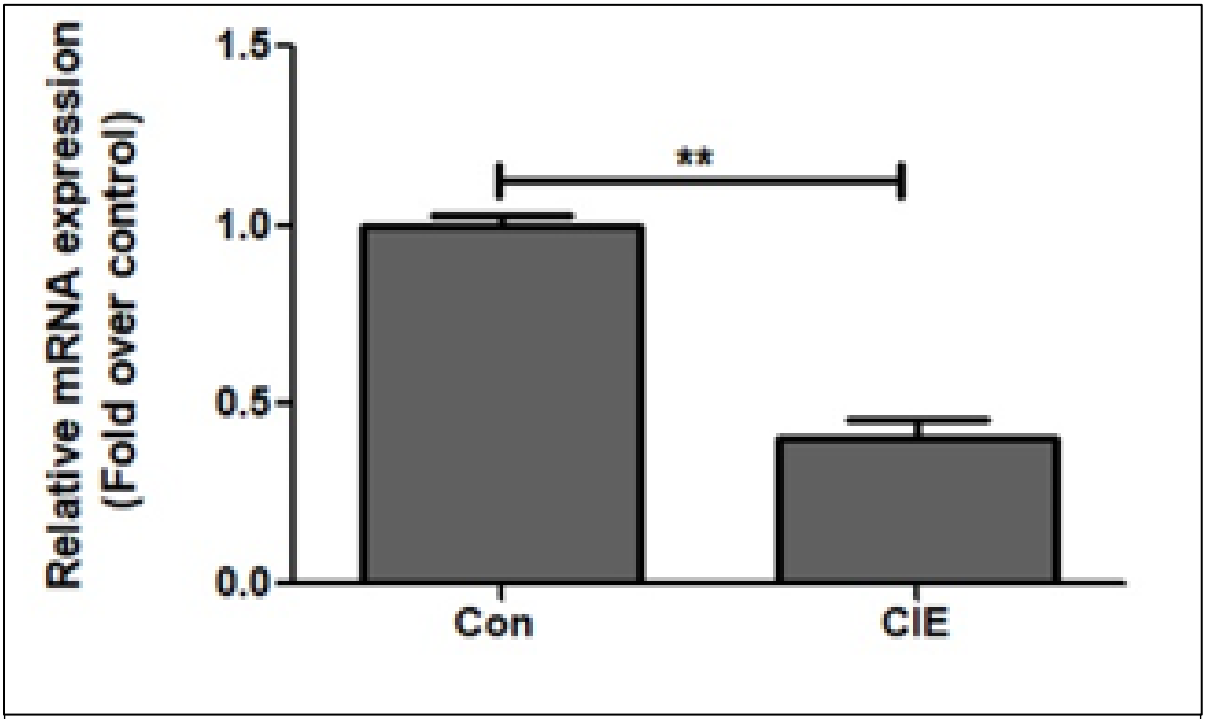

Figure 7: The mRNA expression in the control adipocytes was significantly ($p<0.01$) higher than the expression in CIE adipocytes. The mRNA expression has a direct relationship with MTP expression, indicating less MTP expression in CIE adipocytes (created by student researcher).

Discussion

The mechanisms of MTP in most of the body are well-known, specifically its role in apo-B construction and triglyceride transfer. However, the role of MTP is not well known in adipose tissue. Most recent literature has supported the repression of insulin mediated MTP expression in hepatic cells but no specific relationship in adipose tissue have been identified. Since the 3T3-L1 cells successfully formed lipid droplets, the cells differentiated correctly. Proper differentiation of 3T3-L1 cells is necessary to complete the following steps to the project including chronic insulin treatment, qRT-PCR, and an MTP Activity Assay. Without this crucial step, the cells cannot become insulin resistant and so, no relationship can be confirmed.

Hyperinsulinemia is considered a common factor for diabetes and obesity. Studies have reported that hyperinsulinemia alone is sufficient to cause insulin resistance, which can cause or be caused by an accumulation of lipid droplets and their size (Guilherme et al., 2010). As a result of the 72-hour chronic insulin exposure, lipid droplets formed, grew in size, and accumulated in CIE adipocytes (Figure 3), supporting insulin resistant development.

MTP is essential for apoB-lipoprotein synthesis, transferring both phospholipids and triglycerides. In hepatic cells, MTP activity is downregulated (Higuchi et al., 2011). Like in hepatic cells, triglyceride and phospholipids could not be transported out of the cell regularly. However, this downregulation is much more drastic in adipocytes alone as it may lead to the development of obesity. Since the percentage of triglyceride and phospholipid transfer was less in the CIE adipocytes in comparison to the control, there was less MTP activity in the insulin resistant adipocytes (Figures 5 and 6).

Using a quantitative Real Time PCR, MTP regulation under CIE conditions was verified. The expression of mRNA was also lower in CIE adipocytes, indicating MTP expression under insulin resistant conditions decrease. Therefore, MTP activity under insulin resistant conditions decreases due to a decrease in MTP expression (Figure 7). It is rational to suggest MTP expression and activity play a critical role in adipocyte insulin resistance.

Future Work

Through the elucidation of insulin resistance's role in MTP downregulation, the region in MTP development involved with downregulation, and the transcription factors in MT, steps can be taken to help to make this idea into a reality. Some of these questions are in the process of being answered experimentally through a Dual Luciferase Assay and a Chromatin Immunoprecipitation Assay. This project, as well as future projects, may help in the derivation of new and permanent treatments for obesity for both children and adults in this society.

Conclusions

Many works of scientific literature had similar findings when analyzing 3T3-L1 adipocyte differentiation. Like this project, others have tested 3T3-L1 cell differentiation through Oil Red-O staining and through image analysis, resulting in a successful differentiation of 3T3-L1 cells. The success of the differentiation was expressed through lipid droplet formation and accumulation. However, unlike many experiments, this experiment analyzed the effect of chronic insulin exposure (CIE) on MTP in adipose tissue. This project established that after 72 hours of CIE, 3T3-L1 adipocytes express insulin resistance. This was accompanied by increased lipid accumulation as well as a decrease in triglyceride transfer protein expression. The decrease in MTP expression may be a cause or a consequence of insulin resistance. Since insulin resistance is a leading factor to obesity, bariatric surgery has become a common yet temporary tool to combat this issue. This project is a major step towards obesity prevention and will hopefully contribute to a permanent solution.

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