Biomedical and Health Sciences

The Effects of Chronic Insulin Exposure on Triglyceride Transfer Protein (MTP) Activity and Exposure in Adipocytes

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Research Plan/Project Summary Instructions

The Research Plan/Project Summary should include the following:

A. RATIONALE:

Approximately 40% of the U.S. adult population is obese. Obesity is a major risk factor to the development of insulin resistance and subsequently, diabetes. Insulin is a hormone that facilitates in glucose metabolism. 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, hypertension, and etc (Shanik et al. 2008). Glucose homeostasis is maintained by three bodily locations- liver, adipose, and skeletal muscle. The role of microsomal triglyceride transfer protein (MTP) has only been well studied in the liver. Though not intensely studied in adipose tissue, MTP expression has been discovered in both white and brown adipocytes by Ahamed et al. and Larry L Swift et al. MTP is an essential tool for the synthesis of apolipoprotein-B in the liver and intestine (Dai and Mahmood Hussain, 2012) and facilitates in the transfer of triglyceride, cholesterols, and esters between membrane vesicles. In insulin resistant hepatic cells, there was a decrease in MTP expression, as well as increased triglyceride levels. Since adipose tissue stores triglycerides in lipid droplet form, similar results found in insulin resistant adipocytes may lead to a buildup of fatty acids in the body, resulting in obesity and type 2 diabetes.

B. RESEARCH QUESTION(S) and EXPECTED OUTCOMES:

Research Question:

How does insulin regulate MTP expression and activity in adipocytes?

Expected Outcomes:

MTP expression and activity will decrease in chronic insulin exposed adipocytes in comparison to control adipocytes.

Describe the following in detail:

C. Procedures:

3T3-L1 Cell Culture (put in past tense)

One 3T3-L1 cell vial (ATCC) originated from mice fibroblasts were taken out and put into an ice bucket. Media were made using 500 mL DMEM, 50 mL FBS, and 5 mL of L-glutamine;

sterilization of the media is done through vacuum filtration. The 3T3-L1 vial were then taken out of the ice and 1 mL of media were 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 5 minutes. Once centrifuged, the mixture was taken out, and resuspended. All the content were removed from the Falcon tube and placed into 2 labeled flasks. 12 mL of media was then added to the flask as well. After 48 hours, the cells will be split by removing all the media from the flask and adding 1-2 mL of trypsin. The flask will then be incubated for 30 seconds. To ensure that all the cells are mobile, the flask will be tapped and observed under the microscope. To inactivate the trypsin, 10 mL of media will be added to the flask. The media/cell mixture will be transferred to a 50 mL tube. The 3T3-L1 cells will be centrifuged at 1200 rpm for 5 minutes. The media above the pellet will be removed and 10 mL of media will be added to the pellet and resuspended. 1 mL of the resuspended cells and 9 mL of media will be added to the culture flask, creating a 1 to 10 dilution. The flask will be left in the 4 degree celsius incubator until the cells are 80% confluent. Once 80% confluent, the media in the flask will be removed and 2 mL of trypsin will be added. After waiting 60 seconds, the flask will be tapped to ensure that all the cells are mobile. 8 mL of media will be added to the flask and pipetted up and down. The media/cell mixture will be added to a 15 mL Falcon tube and centrifuged at 1200 RPM for 5 minutes. The top media will be removed and the pellet will be kept. 1 mL of media will be added to the tube and resuspended. Once 24 mL of media is aliquot into a 50 mL Falcon tube, the media/cell mixture will be added into it. 2 mL of this mixture will be added to each well. After 72 hours, the media will be changed.

3T3-L1 Cell Differentiation

After the 72 hour growth period, the 3T3-L1 cells will become 100% confluent and ready for differentiation. The media will be aspirated and 2 mL of the first differentiation cocktail will be administered. The cocktail includes 250 µL of IBMX, 125 uL of Insulin, 25 µL of Dexamethasone, 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 will be administered. This cocktail includes 125 µL of insulin and 24,875 µL of media. 48 hours later, the media will be aspirated and 2 mL of media will be added to each well.

Chronic Insulin Treatment

24 hours after adding media to the transfected 3T3-L1 adipocytes, the first treatment for insulin resistant characterization will be added. The treatment will solely be 500 pM of insulin for half of the plate. 500 pM of insulin will be added again after 24 hours and 48 hours to create CIE adipocytes and control adipocytes. An Oil Red-O staining will then be done to see the difference in lipid accumulation between the control and post chronic insulin exposed adipocytes.

Oil Red-O Staining

An Oil Red-O stock will be created by adding 0.35 g of Oil Red-O dye to 100 mL isopropanol. Using the stock, a working solution will be made by adding 4 mL of deionized water to 6 mL of the stock solution. The media that will be added to the 3T3-L1 adipocytes were removed, and the cells will be washed in a 6 well-plate with PBS three times. Once the cells are washed, 1 mL of the working solution will be added to each well and incubated at 37°C for 15 minutes. Once incubated, the cells will be washed 3 times with PBS, but unlike before, the cells will not be aspirated after the third wash. Images of the stained cells will then be taken with a bright field microscope. 1 mL of isopropanol will be added to each well, the plate will be swirled, and the

plate will be incubated for 5 minutes (Kraus et al. 2016). After incubation, 100 μL of the solution will be added to each well of the 96-well plate and read at 490 nm. The absorbance will read to quantify the lipid droplet content of each well. Images of the stained adipocytes will be taken after 24 hours, 48 hours, and 72 hours.

MTP Activity Assay

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

QT-PCR

Total RNA will be isolated from 3T3-L1 adipocytes by adding 1 mL of TRIzol reagent containing properties, such as phenol, guanidine isothiocyanate, red dye, and etc, to the 6 well plate. With a pipette, the cells will be dislodged from the 6-well plate and will be placed into epindrophs. 250 µL of Trichloromethane (chloroform) will be placed into each epindroph. The epindrophs will then be centrifuged and the supernatant will be removed, leaving solely the RNA in the epindroph. 1.4 mL of 75% Ethanol will be added to each RNA filled epindroph to prevent contamination. Using the NanoDrop™ 2000/2000c Spectrophotometers, the RNA concentration of the CIE and control adipocytes will be determined. The isolated RNA will then be 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 will be used to go forth with the reaction. This will again be used to for the quantitative real time PCR analysis on LightCycler 480, using SYBR Green master mix. The statistical analysis of the qt-PCR will be done using relative changes in gene expression as compared to the reference gene, 18S rRNA.

Risk and Safety:

- NYU-Winthrop Research Lab is a BSA Level 2 Lab
- Goggles and gloves will be used to prevent contamination of any chemicals outside of the laboratory.
- Ethanol will be sprayed on cell plates, flasks,and chemicals when moving in and out of apparatuses.
- Needles and glass will be placed into the hazardous bin.
- All excess solutions will be aspirated into the waste.

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 will be done in GraphPad Prism. A one-way ANOVA will also be done to determine statistical significance ($p<0.05$) of the Real Time PCR results.

D. REFERENCES:

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1. Human Participants Research
    a. N/A
2. Vertebrate Animal Research
    a. N/A
3. Potentially Hazardous Biological Agents Research:
    a. 3T3-L1 cell vials from ATCC
    b. The Biosafety Level 1is based on U.S. Public Health Service Guidelines.
    c. Materials will be placed in hazardous bins and solutions will be vacuumed into waste.
4. Hazardous chemicals, activities & devices:

Chemicals

- DMEM
- FBS
- L-Glutamine
- IBMX
- Insulin
- Dexamethasone
- Rosiglitazone
- Oil Red-O dye
- Deionized Water

- PBS
- Isopropanol
- Ultrapure water
- Buffer K
- PIC
- RT Buffer
- dNTPs
- RT primer
- RNase inhibitor
- MgCl2
- SYBR green

Describe the risk assessment process

- Using the Safety Data Sheet from websites such as Sigma-Aldrich and Fisher Scientific, the risks of each chemical is assessed and acknowledged.

Supervision

- Supervised by Dr. Sujith Rajan. If Dr. Rajan is not available, Ms. Amanda Christiano will be the supervisor. As a lab technician in NYU-Winthrop's research lab, Ms. Christiano is very familiar with all the procedures done by the student researcher.

Safety precautions

- Gloves and Lab coats will be worn at all times. When doing experiments, the student researcher will keep all the materials under the hood. 70% ethanol will be used to sanitize all materials.

Methods of disposal

- All needles and glass will be placed into the Hazardous bin.
- All unnecessary solutions will be aspirated into the waste.
- All other materials (i.e. pipette tips, Falcon tubes, etc.) will be placed in a non-hazardous bin.