The Effects of Global Knockdown of Cytochrome C Oxidase Assembly Protein (Sco2) in Diabetic Kidney Disease

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Research Plan

Biomedical and Health Sciences

A. Rationale:

Diabetes, a chronic disorder that affects millions of people around the world, is becoming an epidemic.[1] In the United States, 30.3 million people have diabetes and by 2030, it is estimated that more than 54.9 million Americans will suffer from it.[2,3] More health care resources are estimated to be spent on diabetes than any other condition since it leads to damage to multiple organs. Diabetes is a lifelong chronic disease caused by high glucose levels. The two most common forms of diabetes disorders are Type 1 and Type 2. Type 1 diabetes is an autoimmune disease caused by the inability of the pancreas to produce sufficient amounts of insulin. Conversely, Type 2 diabetes is a metabolic disorder caused by insulin resistance, in which the cells are unable to use the insulin produced by the pancreas, and glucose is not converted into energy for cells.[4] According to the Center for Disease Control and Prevention, 90% of the diabetic population have Type 2 diabetes.[2] Globally, diabetes is the leading cause of chronic kidney disease with about 40% of patients who are diabetic eventually getting diabetic kidney disease (DKD).[5]

This is a problem because the kidneys are critical organs responsible for maintaining homeostasis by regulating water and electrolyte balance, regulation of calcium, filtering blood, and excretion of metabolic wastes.[6] In DKD, patients will progress to tubular damage and chronic kidney disease. In early diabetic nephropathy, there is a marked increase in mesangial cell number as well as mesangial extracellular matrix. This starts to compress on the endothelial cells, causing the lumen to become smaller in size. Additionally, the basement membrane becomes thicker, podocyte foot process effacement starts to occur, followed by podocyte apoptosis, with a final decrease in podocyte number. This is so detrimental because podocytes are post mitotic cells, meaning they are similar to neurons: once it is lost, it cannot regenerate. The loss of the podocytes allow for proteins to be filtered through the glomerulus which can then finally start causing tubular damage as well.[7]

In addition to maintaining healthy kidneys, since the kidney is a highly metabolic organ, mitochondria are very important for the proper functioning of the kidney. Mitochondria are membrane-bound organelles that maintain a variety of cellular functions, such as the level of reactive oxygen species, making nucleotides, and regulating apoptosis. However, the most important function of the mitochondria is the production of ATP and supplying energy for basal cell function as well as cellular

repair and regeneration.[8] A population of healthy and functional mitochondria is vital for these processes to be carried out. In the setting of diabetes, there is a potential for mitochondrial damage due to a change in substrate availability, increase in reactive oxygen species, and an increase in protein modifications. This can lead to an increased amount of fusion in order to compensate for the increased damaged mitochondria. Unfortunately, there would be many portions of the mitochondria that could become damaged so there could be an even increased amount of fission that would occur. Despite this increase in fission, there is not enough autophagy machinery to keep up with the fission which will lead to more apoptosis. Simultaneously, there will be a demand for new mitochondria, but not enough machinery to make new mitochondria because components were not saved through mitophagy. As a result there will be a decrease in biogenesis. This is problematic due to the importance of mitochondria in kidney function. If there is a decreased amount of mitochondria due to diabetes, then there will be less energy available thus negatively impacting kidney function.[9]

Inherited factors including mutations in genes that impact mitochondrial function and/or substrate delivery may also be important risk factors for DKD. One of those mutations is the Synthesis of Cytochrome C Oxidase (Sco2) protein mutation. Sco2 is an essential protein embedded in the inner mitochondrial membrane. It is a metallochaperone that is essential for the assembly of Cytochrome C Oxidase (Complex IV) because it is involved in the delivery of copper to subunit 1 and 2 to help with the formation of Complex IV.[10] However, when there is a Sco2 mutation present or the Sco2 protein is missing, there is mitochondrial dysfunction. ATP is no longer produced through the ETC because Complex IV is not formed. This mutation is extremely lethal as a decrease is SCO2 results in a Complex IV deficiency thus leading to decreases mitochondrial activity and ATP production.

The current study aims to bridge the gap by observing the Sco2 mutation in diabetic kidney tissue and investigating its effects on kidney function. The primary goal of this study is to determine the effects of a global knockdown of Sco2 in a diabetic injury mouse model on kidney function and to characterize the tubular, podocyte, endothelial damage to determine which cells are reliant on oxidative phosphorylation in the setting of diabetes.

B. Engineering goals:

The objective of this study is to observe the changes that occur between the wild type, db/db, and db/db KI/KI mice. It is hypothesized that the kidney, a highly metabolic organ with an abundance of mitochondria present, and a global knock-down of Sco2 will lead to cell damage in cells that need oxidative phosphorylation for survival in a diabetes model.

C. Experimental Design:

1. **Periodic Acid- Schiff's (PAS) Staining:**

PAS staining helps in detecting PAS polysaccharides such as glycogen, and mucosubstances such as glycoproteins, glycolipids and mucins in tissues. Additionally, within the glomerulus of the kidney, PAS staining also allows us to determine the amount of mesangial expansion, glomerular volume, and tubular injury within the glomerulus.[5] After obtaining slides for WT, db/db, and KI/KI db/db genotypes, PAS staining will be performed in order to determine the effects of the different genotypes on the kidney. The slides will bake for at least 1 hour at 37 °C and cool at room temperature for 10 minutes. Once the slides cool, the hydration process will be initiated. First, the slides will be placed into Xylene bath for 5 minutes twice. Then, the slides will be placed into 100% ethanol, 75% ethanol, and 25% ethanol for 3 minutes each. Due to the hydrophobic wax covering, the decreasing concentration of ethanol will allow for the cells to adapt to the deionized water and not burst on contact. After 3 washes in ethanol, the slides will be placed in deionized water for 5 minutes. After the hydration process, the slides will be immersed into periodic acid for 11 minutes. Next, the slides will be placed in schiff reagent for 15 minutes. Lastly, the slides will be immersed in hematoxylin for 10 seconds per slide. After staining with hematoxylin, the slides will be left in the sink and rinsed in distilled water for 5 minutes. After the completion of staining, the slides will undergo the dehydration process which is simply just the reverse of the hydration process and then coverslipped. Once the slides dry, the tissue will be observed under the bright field microscope and full scan images of the glomeruli and tubules will be taken.

II. **Immunofluorescence staining for podocyte markers:**

A. **Immunofluorescence (IF) for Synaptopodin**

Kidney sections from the 3 genotypes will be stained for Synaptopodin, a podocyte marker. The immunofluorescent staining is a 2 day protocol.

On Day 1, the slides were baked in an oven at 65 °C for at least an hour and then the same hydration process will be used for PAS staining will be applied to the slides. While the slides will be immersed in deionized water, 200ml sodium citrate buffer will be prepared at a 1:10 dilution. After 5 minutes, the slides will be placed into the sodium citrate buffer and then placed in a pressure cooker at 120 °C for 10 minutes. Once the 10 minutes were complete, the slides will be placed in the 4 °C and will be allowed to cool for 30 minutes. During this time, blocking buffer using 2% non-fat milk was prepared with a 3:2 dilution of tris - buffered saline(TBST) to milk. The slides will be taken out of the 4 °C and will be washed in TBST (1:10) for 3 minutes. Then, the blocking buffer will be added to each tissue and the slides will be placed in the 37 °C to block for an hour. As the slides are being blocked, the Synaptopodin primary antibody (goat) will be prepared with a 1:50 in milk. After an hour, the slides will be washed in TBST and the primary antibody placed onto the lisdes and then left to incubate overnight at 4 °C.

On Day 2, the slides will be stained with secondary antibody R*a*G (1:300 dilution in milk). After washing with TBST, sections will incubate with fluorophore-linked tertiary antibody D*a*R 568 (1:300 in TBST). After staining, the slides will be mounted using Prolong Gold antifade mounting media and then photographed using the microscope.

B. **Immunofluorescence for WT1**

Immunostaining will be performed on the kidney using the same method for Day 1 IF staining for synaptopodin (mentioned in the previous section) with the exception of WTI (mouse) primary antibody to target the WTI podocytes markers in the glomerulus.

On Day 2, the slides will be washed in TBST and then fluorophore-linked secondary antibody D*a*M 647(1:300 in TBST). In order to quantify the WT1, we need to count the dapi positive WT1. Dapi stains the nucleus of the cell. In order to stain for Dapi, the tissue will be stained with Hoechst stain (1:1000) and then will be washed in TBST. After staining, the slides will be mounted using Prolong Gold antifade mounting media and then photographed using a microscope. To determine the amount of damage of podocytes within the glomeruli, Dapi + WT1 will be counted for.

III. **Immunofluorescence staining for tubule marker:**

Immunostaining for the Lotus Lectin, a tubular marker for the brush borders of the proximal tubules, will be conducted using the same method for Day 1 as the previous immunofluorescence stainings with the exception of the antibody which was utilized for this staining. Lotus Lectin will be prepared in TBST and placed onto the slides for incubation at room temperature for 10 minutes. After staining, the slides will be mounted with the mounting media, images were taken on the microscope, and the percent area of Lotus Lectin was determined using Image J.

IV. **Immunofluorescence staining for endothelial cells:**

To analyze the differences between the three genotypes in endothelial cells, immunostaining for endothelial nitric oxide synthase(eNOS), an endothelial cell marker, was conducted. The same method will be utilized for day 1 as previously stated, but this staining, eNOS primary mouse antibody was used instead. The primary antibody will be prepared in non-fat milk at a dilution of 1:50. The slides will be incubated overnight at 4 °C. On the second day, the slides will be washed in TBST for 5 minutes and then a secondary antibody, D*a*M 647, will be placed on the slides at a dilution of 1:300. The slides will be incubated for 30 minutes at 37 °C. After 30 minutes, the slides will be mounted with mounting media and images will be taken under the microscope. Using Image J, the percent area stained within the glomerulus will be determined.

V. **Albumin Assay:**

An albumin assay will be performed on the urine samples of the two genotypes, db/db and KIKI db/db, in order to determine the albumin concentration within the urine. The albumin assay consists of 5 major parts: coating, blocking, standards, HRP detection, and enzyme substrate reaction.

A master mix for coating will be created using 1µl of capture antibody diluted with 100µl of coating per buffer. 100µl of master mix will be pipetted into a 96 well plate. The plate will be incubated for 1 hour at 4 °C and then the capture antibody will be removed and plate will be washed 3x with washing buffer. A plate plan will be devised with duplicate wells for each standard and sample. Next, 200µl of blocking solution will be added to each well and incubated for 30 minutes. After incubation, the blocking solution will be removed and the wells will be washed again with wash solution three times each. Using the standard dilution, 8 standards, B-I, will be prepared. 100µl standards, samples of diluted urine, will be transferred into the wells. The plate will be sealed and incubated for 60 minutes. The samples and standards will be removed and wells were washed. 100µl of HRP Detection Antibody was added to each ewell at a dilution of 1:1000 with HRP Conjugate Diluent. The plate will be incubated for an hour and the washed 5x. For the Enzyme Substrate reaction, 100µl of substrate solution(TMB) will be transferred to each well and the plate will be incubated for 15 minutes. The reaction is stopped by applying 100ul of stopping solution to each well. The plate will be read at 450 nm for TMB and the albumin concentration was analyzed.

D. Risks and Safety:

All experimentation will be supervised by the laboratory mentor. In addition, laboratory training will be received on the Stony Brook University Blackboard prior to beginning work in the laboratory. A verbal laboratory training will also be given by the mentor on the first day of work. During experimentation, proper gloves and lab coat will be worn. Any biological waste will be disposed biohazard waste containers.

E. Data Analysis:

Images of the kidney tissue slides will be taken on the bright field light microscope. Quantifications of mesangial area, counting of podocytes, and measurements of glomerular and tubular percent area will be performed by the program Image J. Statistical analysis was done in GraphPad Prism.

F. Post Summary:

No addendums or modifications were made since the completion of this research plan.

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