Hyperglycemia and Mitochondrial Function

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Goal:To determine if acute exposure to abnormal glucose levels induces mitochondrial fission and endothelial dysfunction through increased Drp1-Fis1 activity in humans.

Rationale: While DM often includes multiple metabolic abnormalities, the central phenotypical attribute of DM is abnormal systemic glycemic exposure. Hyperglycemia and hypoglycemia (occurring secondary to therapies), both acutely and chronically, are critical to both chronic and acute-on-chronic endothelial dysfunction in DM.[1-9] Both exposures rapidly impair human vascular endothelial dysfunction through increased oxidative stress, inflammation, and a loss of NO bioavailability in both healthy non-DM subjects and patients with DM. [5-10] Hyper- and hypoglycemia driven mitochondrial ROS production critically contribute to the development of endothelial dysfunction.[4, 11] *However, the mechanisms behind the acute phenotypical change in the endothelium with acute hyperglycemic exposure remain poorly understood.* Better understanding the mechanisms underlying the adverse impact of acute dysglycemia on the endothelium could lead to novel treatments to mitigate the burden and complications of CV events in patients both with and without DM.[12-15]

Hyperglycemia also drives over-expression of Drp1 and Fis1 in the endothelium.[16] Drp1 and Fis1 interact to drive mitochondrial fission leading to subsequent excessive mitochondrial superoxide production.[16, 17] Molecular suppression of Drp1 or Fis1 blocks hyperglycemia's adverse impact cultured endothelial cells.[16] Our preliminary data show that Drp1 and Fis1 also control low glucose driven mitochondrial fission and excessive mitochondrial ROS production (Fig X or not shown). While there are multiple docking proteins for Drp1 on the outer mitochondrial membrane besides Fis1 (Mff, MiD49, MiD51), Fis1 is the preferred Drp1 docking protein in the setting of cellular stressors, including high glucose, in cell culture.[18-21] These foundational data and our preliminary data strongly support our initial strategy of concentrating on Drp1 and Fis1.

In this study, we will test the hypothesis that acute *in vivo* exposure to hyperglycemia increases mitochondrial network fragmentation and mitochondrial ROS production in human arterial endothelial cells. Affirmative findings will critically establish that acute *in vivo* exposure to clinically relevant hyperglycemia levels in humans triggers mitochondrial fission and excess mitochondrial ROS production. Further, affirmative findings will establish that inhibition of mitochondrial fission proteins Fis1 and Drp1 reverses acute dysglycemia-related endothelial dysfunction.

Table 1: Inclusion/Exclusion Criteria for Aims 1 and 2

T2DM Inclusion Criteria

1. Adult age 21-70
2. If on HMG-CoA reductase therapy, must be on a stable dose for at least 6 weeks prior to enrollment
3. Diagnosis of type 2 diabetes by a physician as defined by the American Diabetes Association standard criteria

Inclusion Criteria for Healthy subjects

1. Adult age 21 to 70
2. No evidence of metabolic syndrome or diabetes, hypertension (BP≥140/90), or high cholesterol (LDL≥160) at the time of screening.

T2DM Subject Exclusion Criteria:

1. History of stroke, peripheral arterial disease, or coronary artery disease (as defined by the presence of at least one coronary stenosis ≥ 40% on angiography or by confirmed history of myocardial infarction by standard criteria). History of bleeding disorders.
2. Evidence of other evident major illness including chronic renal insufficiency(plasma creatinine > 1.4 for women or 1.5 for men), liver disease (AST or ALT greater than 2.5 x normal), and cancer currently undergoing therapy or had therapy for cancer within 1 year of enrollment.
3. Pregnancy as determined by urinary beta-HCG test
4. Thienopyridine, anti-thrombin/Xa, or warfarin therapy at time of screening

Exclusion Criteria for Healthy Subjects:

1. Meet any of the exclusion criteria for diabetic or metabolic syndrome group
2. Meet criteria for type 2 diabetes or metabolic syndrome
3. On medication for cholesterol or blood pressure
4. History of superficial thrombophlebitis, deep vein thrombosis and pulmonary embolism
5. Women of childbearing potential using systemic birth control

Protocol

Subjects will be recruited according to our inclusion/exclusion criteria listed in **Table 1**. We employ a multifaceted approach to recruitment, including flyers, community newspaper ads, internet ad postings, and direct recruitment from the Internal Medicine Clinics of Froedtert Memorial Lutheran Hospital/Medical College of Wisconsin which saw approximately 50,000 unique outpatient visits over last year. The electronic health record is leveraged to identify potential subjects by HIPAA guidelines and MCW policies. .Potential subjects will undergo phenotyping that includes a detailed medical history, anthropomorphic measurements, blood pressure and heart rate measurements, and a blood draw for measurements that include fasting lipids, glucose, and glycosylated hemoglobin, creatinine, and liver function tests. Healthy, non-DM subjects who pass the screening as per the inclusion/exclusion criteria in **Table 2** will be enrolled in the study protocol.

Following an overnight fast (12 hours), subjects will come in the morning to our Adult Translational Research Unit (A-TRU) which is part of the Clinical Translational Research Initiative of Southeast Wisconsin (8UL1TR000055). An antecubital intravenous line will be placed to facilitate blood glucose sampling during the hyperglycemic challenge. An antecubital intravenous catheter will be placed to facilitate obtaining endothelial cells from the vein by J-wire biopsy for of mitochondrial testing. The PI has published experience with the J-wire endothelial cell biopsy technique, and the technique has been extensively validated technique.[22-27] An initial venous glucose sample will be taken from the antecubital vein and an initial J-wire biopsy of the radial artery endothelium will be performed through the radial arterial line. Subjects will then be asked to drink a standardized 75 g glucose drink created by the A-TRU nutritionist- a standard oral glucose challenge as used clinically. Blood glucose samples will be taken hourly after that until 2 hours post drink ingestion. At one and 2 hours post-ingestion, J-wire endothelial biopsies of the radial artery will be repeated. Three separate J wires will be passed into the radial artery for each measurement time point (0,1, and 2 hours).

Because of the prolonged fast, subjects may have a decrease in blood sugar to <70 during this study. To mitigate this phenomenon, subjects will be given 6 ounces of juice to drink at the 2-hour mark. If subjects report symptoms of hypoglycemia, which include but are not limited to feeling tremulous, headache, lightheadedness, feeling clammy or sweaty and nausea, the blood glucose level will be checked via a fingerstick. If the blood glucose level is < 70, they will be treated with juice and/or food. The fingerstick will also be repeated periodically to ensure adequate response to the intervention.

In non-DM subjects, the 75 g oral challenge induces a peak increase in systemic glucose at 1-hour post-administration at which time endothelial dysfunction is concomitantly detectable.[28] While we could achieve a greater degree of hyperglycemia for a longer period using a hyperinsulinemic-hyperglycemic clamp procedure, the hyperinsulinemic-hyperglycemic clamp procedure fails to induce endothelial dysfunction, likely secondary to the pharmacological levels of insulin required for the protocol.[29] Therefore, the oral glucose tolerance test is better suited for the purposes of this Sub-Aim.

A portion of the endothelial cells isolated from the J-wires will be used to measure mitochondrial network complexity prior hyperglycemic challenge, 1-hour post challenge, and 2 hours post challenge. We will visualize the mitochondrial networks in these cells using our previously reported and validated immunofluorescence method using cytochrome c antibodies to tag the mitochondria.[27] Network fragmentation (high number=greater fragmentation and fission) will be quantified by network fragmentation count calculated with ImageJ (NIH, Bethesda) using a validated protocol adapted in collaboration with Dr. Jalees Rehman from the University of Illinois-Chicago.[30, 31]

SUB-STUDY: The J-wire procedure proposed in this protocol is used to isolate endothelial cells and used to measure mitochondrial network complexity. In order to train (new) study staff on this procedure, as well as refine the best way to isolate these cells off the wires after they have been obtained from a subject approximately 20 subjects will be enrolled into a "sub-study" under this protocol. In this sub-study, subjects will sign informed consent one or two antecubital IV lines will be paced. The Research Fellow or PI will obtain endothelial cells by passing 1 to 3 J-Wires through the IV. No fasting is required of the subject and we will not need to collect any medical history on the subject. Subjects in this sub-study could enroll in the main study if desired, they would be consented again with the main study consent.

PRELIMINARY DATA: Our published work supports the feasibility of measuring mitochondrial network fragmentation in endothelial cells captured by J-wire biopsy.[16]

Mitochondrial superoxide production using MitoSox™(Life Technologies). Cells are re-suspended in PBS buffer and incubated for 30 minutes at 37ºC with 5 μM of MitoSox™ (Invitrogen), a fluorescent probe specific for mitochondrial superoxide production. Cells are then immediately fixed with 2% paraformaldehyde and plated on poly-lysine coated slides (Sigma, St. Louis) and allowed to settle. Following the addition of DAPI (4', 6'-diamidino-2-phenylindole hydrochloride) for nuclear identification to help discern endothelial cells from polymorphonuclear cells, fluorescence intensity of endothelial cells are measured by fluorescence microscopy (Nikon E55i). Images are analyzed using MetaMorph 6.1 (Universal Imaging). Fifteen to twenty cells are measured, and the average fluorescence intensity reported for each sample. All images are captured with the same exposure time. Values are reported ratios of subject fluorescence to that of human umbilical vein endothelial cells (HUVECs) stained simultaneously for

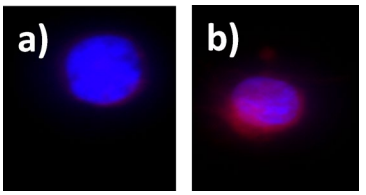

Figure 2: Sample endothelial cells taken from a human volunteer after 60 minutes of euglycemia (a) and hypoglycemia (b). Red- MitoSox™, Blue- DAPI.

interexperimental normalization.[23, 24, 32] **PRELIMINARY DATA:** We have successfully obtained vascular endothelial cells using the J-wire technique and measured mitochondrial superoxide production prior to and following lowering blood glucose levels for 1 hour, proving the feasibility of these measurements (**Figure. 2**).

Data Analysis and Sample Sizes: The primary outcome for this sub-aim is the measurement of mitochondrial network complexity from before oral glucose administration to 1 hour afterward measurements. These will be compared by paired t-tests with log transformation for normality or non-parametric methods if needed. A secondary analysis using repeated measures ANOVA will also be performed using data from all 3 time points. Marginal/conditional residual diagnostics and influence diagnostics will be performed. If some of them are violated, log-transformation or non-parametric methods will be considered. Sample size calculations for mitochondrial network complexity measurements were derived from our observations of HUVECs described in our preliminary data section. The mean difference in network fragmentation in the preliminary data was 1.5±0.8 between normal glucose and high glucose exposure. Power analysis was performed for paired test and repeated measures ANOVA assuming 30% inflation in the number need to account for dropouts and failed J-wire captures. A mean difference between before oral glucose administration and 1 hour afterward measurements in network complexity was assumed to be 40% lower than our preliminary data in light of our less aggressive *in vivo* hyperglycemic exposure and the same standard deviation. Also, 4 hour afterward measurements were assumed to be back to the level of prior oral glucose administration. The correlation between time points was assumed to be 0.5. Then, we will need to recruit 15 subjects to have at least 90% power at $\alpha=0.05$ for paired t-test and repeated measures ANOVA. . Mitochondrial superoxide measurements are a secondary outcome whose results will be analyzed in the same manner as network fragmentation.

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