

## Statement of Purpose

### Study Rationale.

Diabetic retinopathy (DR) is one of the microvascular complications of diabetes mellitus (DM). The majority of patients with DM eventually develop some degree of DR<sup>1,2</sup>. Despite the appreciation that controlling blood sugar is protective<sup>3</sup>, many people with DM still advance to DR. Mitochondria dysfunction is associated with diabetic retinopathy pathogenesis<sup>4,5,6</sup>. Millions of people would benefit from additional approaches to prevent/delay DR.

This proposal seeks to overcome existing roadblocks that prevent us from developing approaches to prevent/delay DR. We will accomplish this by identifying novel biomarkers, i.e. processes that protect the retinal vasculature from advancing to retinopathy. Such biomarkers will improve our current ability to diagnose DR. They will also be instrumental for assessing efficacy of new therapies; this is an essential component of the drug development process.

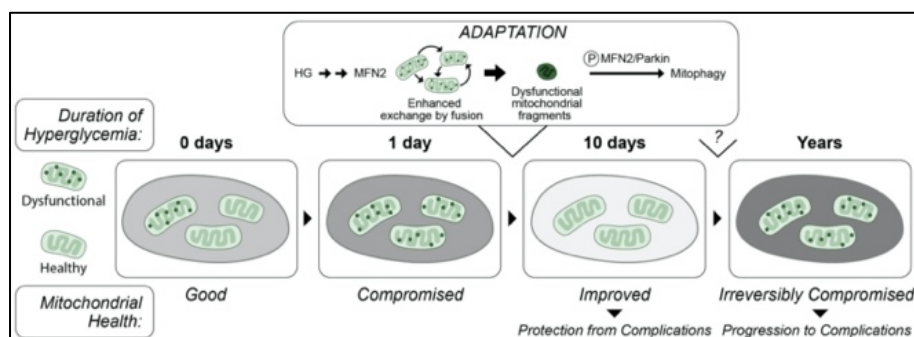
This proposal will also provide the conceptual foundation to develop new therapeutic approaches to prevent/delay DR. As outlined below, we discovered that cells within retinal vessels can resist DM-induced death. The underlying mechanism involves mitochondrial adaptation. The goal of this project is to identify processes that induce mitochondria adaptation. This information will birth a new therapeutic strategy, namely enforcing and/or restoring mitochondrial adaptation as an approach to prevent patients with DM from developing DR.

### Clinical Relevance

Diabetic retinopathy is a significant disease that has a need for improved therapeutics beyond anti-VEGF and laser therapies. Clinically detectable vascular pathology is a relatively late manifestation of diabetic retinopathy. Understanding mechanisms that could further delay development of this component of the disease, as this study designed to do, are important. We will accomplish this by identifying novel biomarkers and mechanistic insights regarding the well-known but under-studied processes that protect the retinal vasculature from advancing to retinopathy in patients with diabetic retinopathy.

### Hypothesis

The overall hypothesis is that soon after the onset of DM, retinal vessels undergo adaption and thereby protects them from succumbing to retinopathy, which occurs only after loss of such adaption. In the course of this study we hypothesize that high glucose (HG) increases oxidative stress and triggers processes (e.g. mitophagy), which enhance the functionality of mitochondria.



**Fig. 1: Hyperglycemia-induced mitochondrial adaptation (HIMA) is a plausible explanation for why diabetic retinopathy always takes years to develop.**

### Study objectives

- 1) Characterize HIMA.
- 2) Test if mitophagy, a process that eliminates dysfunctional mitochondria, is necessary and/or sufficient for HIMA.

## ▪ Preliminary data

The preliminary studies described below are the basis for this aim's working hypothesis that HG increases oxidative stress and thereby triggers processes (e.g. mitophagy), which enhance the functionality of mitochondria. One of this aim goal's is to determine whether the consequence of such adaptation is beneficial to cells; does it suppresses the inflammation index to survive DM-associated stress?

### Hyperglycemia induces mitochondrial adaptation

The current dogma regarding HG-induced oxidative stress is that it drives progression of DR. We utilized powerful tools (roGFP sensors) and developed novel assays (TBH challenge assays; Fig 2) to interrogate the effect of hyperglycemia on the redox status of primary human retinal endothelial cells (HRECs).

The four salient features of these sensors are 1) they are GFP variants whose fluorescence reflects the oxidative status, 2) they are tagged to localize them to a specific subcellular compartment 3) they are fused with a domain that imparts preference for a specific branch of the redox system and 4) they are reversible and thereby enable observation of not only an increase, but also a subsequent decrease in oxidative stress in live cells and in real time<sup>11</sup>.

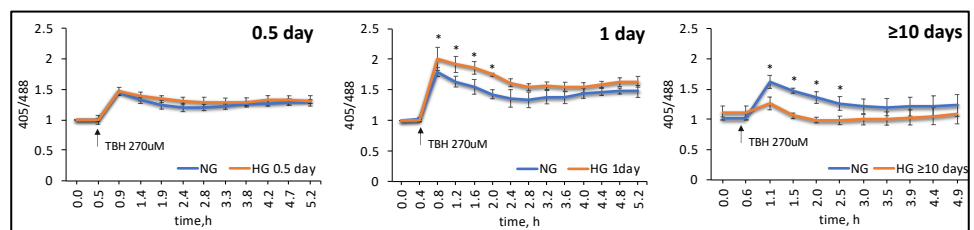
We discovered that while the entire cell was exposed to HG, its effect was not uniform across subcellular compartments; the level of ROSs was altered by HG in only a subset of subcellular compartments. After 10 days of HG, the oxidative stress was elevated in the plasma membrane/Golgi (data not shown). Learning which subcellular compartments are affected is important because the consequences are not uniform for all compartments. For instance, increased oxidative stress within the cytoplasm activates KEAP1/NRF2 and results in altered expression of NRF2-regulated genes. While elevated basal oxidative stress in the plasma membrane resonates with the known ability of HG to increase the generation of ROS in the plasma membrane (by activating PKCs and NOX)<sup>7,8</sup>, the paucity of an effect on the mitochondria was a surprise (data not shown) because other have described multiple mechanisms by which HG perturbs redox homeostasis in this organelle<sup>9,10</sup>.

The roGFP sensors allowed us to extend our analysis beyond the basal level of ROS species, and we did so by assessing the redox capability of the mitochondria. To this end we developed the TBH (tert-Butyl hydroperoxide) challenge assay in which a bolus of TBH is added and the subsequent change (rise and resolution) of ROS species in the mitochondria is monitored. As shown in Fig 2, TBH caused a rapid rise in the level of H<sub>2</sub>O<sub>2</sub>, which subsequently declined somewhat. While the response of cells exposed to HG or NG (normal glucose) for 0.5 days was comparable, after 1 day, the TBH-induced increase of H<sub>2</sub>O<sub>2</sub> was higher and persisted longer in the HG- versus NG-treated cells (Fig 2).

Curiously, increasing the duration of the exposure to HG reverses this effect. After 10 days, the response of NG cells was unchanged, whereas the HG cells improved; TBH induced a smaller rise and faster resolution of H<sub>2</sub>O<sub>2</sub> (Fig 2). These data demonstrate that HG induced adaption of the mitochondria: their ability to cope with an acute oxidative insult improved. Adaption was not observed for any of the other subcellular compartments, which reinforced the subcellular-specific effect of HG that we noted monitoring basal oxidative stress.

These discoveries were possible because of the unique capabilities of roGFP sensors (real time and reversible), and novel assays that we performed with them (TBH challenge). The use of powerful tools and novel assays is a plausible explanation for why HIMA has not been discovered by others who investigated HG-induced oxidative stress.

**Fig. 2: The duration of exposure to HG influenced the mitochondria's capacity to restore redox homeostasis following a TBH (tert-butyl hydroperoxide) challenge.**



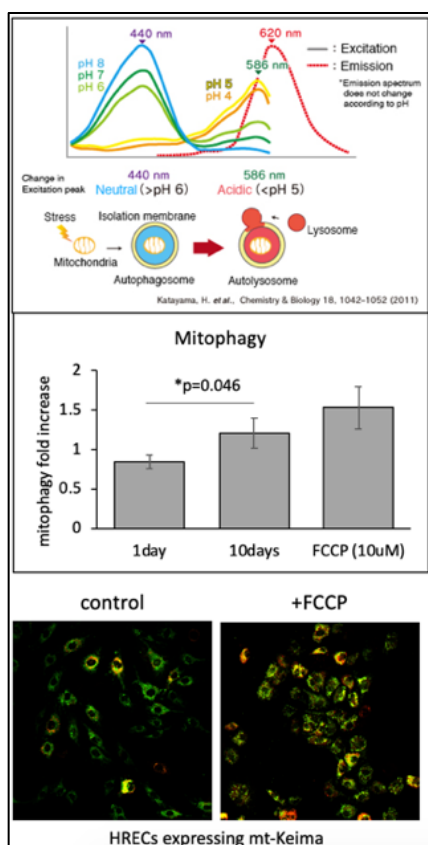
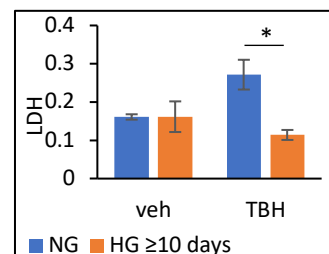
HRECs expressing the mito-H<sub>2</sub>O<sub>2</sub> redox sensor were exposed to either NG, or HG for the indicated duration. The oxidative stress due to H<sub>2</sub>O<sub>2</sub> was measured before and after addition of TBH (arrow) for approximately 5 hrs. The resulting data were normalized to  $R_{red}$ <sup>12</sup> and expressed as a fold change over

vehicle. The graphs show the average  $\pm$ SD for 4 independent experiments. The asterisks indicate time points at which differences between NG and HG were statistically significant.

### Adaptation improves the viability of cells

We assessed the effect of adaptation on viability; i.e. the ability to resist death in the face of DM-associated oxidative stress. Using a commercially-available kit that measures LDH (lactate dehydrogenase) activity – a measure of the integrity of the plasma membrane and hence, the cell viability. We compared basal (vehicle) and stress-induced death in NG and HG-HRECs. HG-treated cells that undergone adaptation were more resistant to TBH - induced cell death (Fig 3).

**Fig. 3: HG treated cells are more resistant to TBH-induced death.** Cell death was assessed by quantitative measurements of LDH that is being released into the media when the plasma membrane is damaged. HRECs were treated with vehicle (veh) or 5mM TBH for 4 hrs for stress-induced cell death. The experiment was repeated  $n=3$  times in triplicates per condition and graphed as the mean  $\pm$ SD.



### Adaptation is associated with increased mitophagy

Pre-conditioning, a brief exposure to a sub threshold level of injury, protects organs such as the brain and heart from subsequent insult. Pre-conditioning involves changes in the mitochondria, which included increased mitophagy, a process that eliminates dysfunctional mitochondrial<sup>13,14</sup>. Because autophagy/mitophagy contributes to the beneficial effects of pre-conditioning<sup>15,16</sup>, we considered if mitophagy was involved in HIMA. To this end we established an approach to quantify mitophagy, and used it to discover that adaptation is associated with increased mitophagy (Fig 3). Furthermore, we learned that the mRNA expression level of mitochondria quality control regulators (PINK1, OPTN – mitophagy, and TFAM – biogenesis, i.e. de novo synthesis of mitochondria) increased as cells underwent adaptation (data not shown).

**Fig 3: Mitophagy increased as cell undergo adaptation.** The text above describes the diagram to the left. The two photos are of HRECs that stably express mt-Keima, which were treated with either vehicle (control), or FCCP (carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazine) to induce mitophagy. The bar graph shows the mean  $\pm$  SD of  $n=3$  independent experiments.

### Identification of differentially expressed genes (DEGs) in HRECs treated with HG.

HRECs exposed to 1 day and 10 days of either NG, or HG were subjected to RNAseq. The resulting data were analyzed by two-way ANOVA test to determine DEGs between 1) NG vs. HG 1-day 2) NG vs. HG 10-days. We will use this dataset to determine the effect of adaptation on the inflammation index outlined in Aim 1.

### Summary

- The effect of HG is compartment-specific
- HG induces mitochondrial adaptation, which improves the functionality of the mitochondria
- Adaptation is associated with increased mitophagy

## Research plan

### ▪ Background

*Pathogenesis of DR (diabetic retinopathy) is incompletely understood*

The risk factors associated with developing DR include duration of DM (diabetes) and magnitude of HG (hyperglycemia)<sup>1, 2</sup>. Result from animal studies and analysis of donor tissue demonstrates that DM elevates oxidative stress within the retina<sup>17</sup>. HG increases oxidative stress in cultured cells (including retinal endothelial cells) in both the cytoplasm, and mitochondria<sup>7-10</sup>. The initial rise of ROS within the mitochondria damages mitochondrial DNA (which encodes the enzymes responsible for the electron transport chain) and thereby results in electron transport dysfunction, which further elevates oxidative stress within the mitochondria.

The current dogma regarding HG-induced oxidative stress is that it drives progression of DR. A gap in our understanding of DR pathogenesis is an explanation for why DR takes so long (decades) to develop. Such information will be valuable in multiple ways. It will improve our current ability to diagnose patients and evaluate new therapies. It will also guide approaches to delay/prevent DR, which is currently not possible.

*The mitochondria promote viability/functionality of cells in adverse conditions*

Recent studies have elucidated the role of mitophagy as a protective response to inflammatory stress in both human and rodent  $\beta$  cells. Diabetogenic proinflammatory cytokines induced mitophagy, which further promoted  $\beta$  cell survival and prevented diabetes by countering inflammatory injury<sup>18</sup>. In additional organs, the exercise capacity of skeletal muscle is dependent on autophagy to clear dysfunctional mitochondria. As one of the mechanisms of pre-conditioning, it was driven by the REDD1/TXNIP complex, which increases oxidative stress and thereby promotes autophagy of mitochondria<sup>14</sup>. Similarly, ischemic post-conditioning of the retina improves the retina's neural function and is dependent on autophagy, which also strongly suggests a mitochondrial involvement<sup>15</sup>. Together these findings indicate that mitochondria adapt to environmental conditions in ways that protects from pathology.

There are some publications investigating DM/HG and the concept of pre-conditioning. Patients with T1D are protected from ischemia-induced injury of skeletal muscle<sup>19</sup>. Similarly, one week of DM protected mice from ischemia/reperfusion-induced heart injury as compared with non-DM mice<sup>20</sup>. The underlying mechanism of this phenomenon has not addressed. As compared with ischemia or hypoxia, HG-induced pre-conditioning is a largely unexplored area of research.

*DR pathogenesis is associated with dysfunctional mitochondria*

There is compelling evidence that mitochondrial dysfunction is associated with pathogenesis of DR. DM upregulates mitophagy in the retina<sup>4</sup>, and primary Müller cells and retinal pigmental epithelial cells *in vitro*<sup>4, 21</sup>. As diabetes duration increases, an uncoupling of the rate of mitochondrial biogenesis and rate of clearance of dysfunction of mitochondria is responsible for DR in experimental animals; such aberrant mitochondrial quality control is also observed in patients with DR<sup>4</sup>. Pre-treatment with mitophagy-inducing agent NGR1 prevented DR via PINK1-dependent enhancement of mitophagy<sup>22</sup>. Pericyte loss and capillary drop out may also be driven by mitochondrial dysfunction since mitochondrial leakage (a hallmark of dysfunction) increases susceptibility of cells to apoptosis. Furthermore, mitochondrial disintegration/fragmentation activates NLRP3-mediated production of inflammatory cytokines and necroptosis<sup>23</sup>. Finally, mitochondrial dysfunction-driven death of vascular cells is likely to compromise the vasculature's barrier function, triggering a self-perpetuating cycle of leakage and inflammation. These concepts support the idea that loss of HG-induced mitochondrial adaptation set the stage for developing DR (Fig 1).

### ▪ Study design and methods

Throughout this project we will continue to use 5 and 30 mM glucose for our NG and HG conditions, respectively. The normal fasting level of glucose is 4-6 mM; greater than 7 mM is considered diabetic. The blood sugar of patients with unregulated DM can rise to 30 mM and beyond; a concentration of 25 or 30 mM glucose is used in the vast majority of in vitro models of DM. Specific approaches to accomplish each aim of this study are outlined in more detail below.

#### **Aim 1 Characterize HIMA.**

**Assess mitochondrial functionality.** We will determine the functionality of the mitochondrial before and after they adapt, i.e. after 10-days exposure to either NG or HG. To this end, we will quantify

established parameters such as oxygen consumption rate (OCR), ATP generation, NADPH/NADP ratio and mitochondrial membrane potential. Additional parameters will be monitored as needed. The OCR and ATP generation will be quantified using the Agilent Seahorse XF Cell Mito Stress Test Kit and XF Real-Time ATP rate assay, respectively, with a SeaHorse Xf96 instrument, which is available within the flow cytometry core facility of the UIC RRC (Research Resources Center). The results of OCR will inform us of mitochondrial uncoupling that may potentially be responsible for redox adaptation and guide aim 3 in identifying genes. The NADPH/NADP ratio and mitochondrial membrane potential will be determined using commercially available kits (Abcam; ab65349 and ab113850, respectively).

**Determine the effect of adaptation on the inflammation index.** We will compare the inflammation index before and after cells adapt. First, the data from RNAseq DEGs analysis will be subjected to Ingenuity pathway analysis (IPA, unbiased), and GeneOntology (inflammation-specific) in order to identify inflammation pathways affected by HG. The inflammation index will include the following types of genes/proteins (we will modify this list as needed): *Inflammasome activation*: NLRP3, ACS, caspase 1, IL-1b, TLR4, SIRT1; *Cytokines and their inducers*: VEGF, EPO, TNFa, Ang2, IL-5, IL-6, IL-8, IL-13, TGFbs, iNOS, COX2, NFkB; *Recruitment of immune cells*: CCL2, CCL20, CXCL12, ICAM1, VCAM1.

We will quantify the level of mRNA expression for most of the inflammation index by qRT-PCR. Inflammasome activation results in maturation of IL-1b and IL-18 protein. Consequently, ELISA will be used to quantify the level of processed/mature forms of IL-1b and IL-18 in the cell lysate and conditioned medium of cells. Finally, activation of transcription factors such as NFkB involves translocation from the cytoplasm into the nucleus. This readout will be done by microscopy of cells stained with a fluorescent nuclear stain (such as DAPI) and a NFkB antibody with a fluorescent tag. The extent of colocalization, which can be quantified, indicates activation. We performed these types of analyses in the past<sup>12</sup>; our Departmental microscopy core facility has both the equipment and personnel needed to accomplish this aim.

We will use the results from the viability studies described above to guide our pilot experiments in this series of experiments. We will first determine conditions that elevate the inflammation index of NG cells and identify those members of the panel that are most informative. We will then use these conditions to test our working hypothesis that exposure to HG at first elevates the inflammation index (1 day of HG) and then reduces it at 10 days of HG treatment (Fig 1). Furthermore, we will determine if the timing of such a reduction concurs with adaptation.

## **Aim 2 Test if mitophagy, a process that eliminates dysfunctional mitochondria, is necessary and/or sufficient for HIMA.**

**Determine the role of mitophagy in adaption.** Fig 3 both illustrates the approach that we will use to quantify mitophagy, and demonstrates that increased mitophagy is associated with adaptation. To determine if mitophagy is required for adaptation we will use a combination of molecular and pharmacological approaches. More specifically, we will use siRNA-mediated approach to suppress expression of genes that are required for mitophagy (e.g. MFN2, OPTN), and compare it with scrambled siRNA control. The resulting cells will be characterized for expression of MFN2 and OPTN (mRNA and protein) and their ability to undergo mitophagy in response to FCCP. As compared with the scrambled-siRNA transfected cells, the MFN2- and OPTN-siRNA cells should have lower levels of the target gene and a reduced ability to undergo mitophagy. We will then test the capacity of cells that have impaired mitophagy to undergo HIMA, i.e. if HG improves their ability to reestablish redox homeostasis following a TBH challenge (as in Fig 2).

Finally, we will test if increasing mitophagy is sufficient to induce adaptation in NG cells. To this end we will use the pharmacological approach: Torin2, SR3677 and Urolithin A (UA). While all three agents induce mitophagy, UA's FDA-approved status increases the translational potential of this line of investigation. We will perform pilot experiments to determine the minimal dose of drugs that induces mitophagy, and then assess if increased mitophagy is sufficient to drive adaptation. Furthermore, the mechanism of action of a drug will point out to the mechanism of mitophagy induction by HG. Our final conclusion regarding the requirement for mitophagy in HIMA will encompass the result of both of these experimental approaches.

The outcome of this series of experiments will reveal if enhanced mitophagy is necessary and/or sufficient for adaptation.

- **Statistical Plan**

All experiment will be run in triplicates on at least 3 independent occasions. Statistical tests such as the student t-test will be used to determine if differences between experimental conditions are statistically significant.

- **Anticipated Results**

We expect that HG-induced changes in the expression of genes triggers mitophagy which is required for HIMA (Fig 1). Consequently, inhibiting mitophagy either pharmacologically or molecularly will prevent adaptation. Similarly, UA-induced mitophagy will improve mitochondria functionality and hence, the response to oxidative stress challenge. The results of this series of experiments will rigorously test the working hypothesis presented in Fig 1.

- **Pitfalls and Alternative Approaches**

In this course of this aim we expect to both elucidate the underlying mechanism of HIMA, and identify its diagnostic features.

While DM increases oxidative stress throughout the retina, this proposal is focused on ECs because this is a reasonable starting point and an appropriate scope for the project. Because ECs are the first to encounter hyperglycemia, and have a unique metabolism (glycolysis instead of oxidative respiration even when oxygen is not limiting)<sup>20</sup>, the response of the ECs to HG may not be generalizable. Consequently, glomerular endothelial cells will be investigated in future studies.

We may find that changes in the mitophagy that are associated with HIMA, do not provide a full mechanism of HIMA. This would be valuable information that will guide the direction of our next steps. For instance, mitochondria membrane uncoupling is implicated in activation of mitophagy<sup>24</sup>. The assessment of mitochondria functionality studies using OCR outlined in aim 1 will inform us. We will use the types of molecular and pharmacological approaches described in this aim to investigate the effect of HG on mitochondria uncoupling, and the importance of such changes on acquisition of HIMA.

We may learn that not all mitophagy inducing drugs are sufficient to drive HIMA. That will point out the mechanism of mitophagy induction by HG.

- **Timeline**

We will complete the proposed studies within 12 months.

- **References**

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