Hyperglycemia Promotes Mitophagy and Thereby Mitigates Hyperglycemia-Induced Damage

Anara Serikbaeva, Yueru Li, Balaji Ganesh, Ruth Zelkha, and Andrius Kazlauskas

From the Departments of Physiology and Biophysics and Ophthalmology and Visual Sciences and Research Resources Center, University of Illinois at Chicago, Chicago, Illinois

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Address correspondence to Andrius Kazlauskas, Departments of Ophthalmology and Visual Sciences and Physiology and Biophysics, University of Illinois at Chicago, 1905 W. Taylor St., Chicago, IL 60612.
E-mail: ak20@uic.edu.

Almost 10% of our planet’s population has diabetes mellitus (DM); its incidence is increasing, and we have been unable to curb this trend. People with DM develop complications that reduce their quality of life. For instance, diabetic retinopathy (DR) is the leading cause of blindness in working-age individuals.

In addition to our inability to prevent DM, approaches to protect people with DM from succumbing to DR are ineffective; most patients with DM still eventually develop DR. The only DR prophylactic is controlling blood sugar, which is fraught with noncompliance and ineffectiveness—even some diligently compliant patients develop DR.

The prevailing understanding of what causes DR (which includes self-amplifying oxidative stress—induced damage) predicts that it will develop soon after the onset of DM. Yet, this is not the case; DR develops only after many years of DM. Furthermore, some patients with DM do not develop complications (including DR) for ≥50 years, and such resistance is unrelated to glycemic control. These clinical observations suggest the presence of an endogenous system that protects from the deleterious effects of DM. The existence of such a system remains speculative, and the reason for the delay in the onset of DR in patients with DM is unknown.

Mitochondria dynamics ensure that the quality and capability of the mitochondria are in sync with the environment of the cells. This process involves fusion and fission that mixes and redistributes the mitochondrial...
contents to sequester dysfunctional mitochondria, which are eliminated by mitophagy. Mutation of key governors of mitochondrial dynamics either causes or is associated with diseases that afflict a large percentage of the world’s population (e.g., neurodegenerative disease, diabetes, retinopathies, cancer, and cardiomyopathies).17,20–22

In this article, we describe the discovery of a high glucose (HG)—induced system that protects retinal vascular cells from the damaging effects of HG.

Materials and Methods

Tissue Culture

Primary human retinal endothelial cells (HRECs) were purchased from Cell Systems (ACBRI 181; Kirkland, WA). They were isolated from donor A, a 26-year-old White man. Cells were authenticated for cytoplasmic von Willebrand factor/factor VIII, cytoplasmic uptake of Di-I-ACLDL, and cytoplasmic CD31, glial fibrillary acidic protein, NG2, and platelet-derived growth factor receptor-β by immunofluorescence. Mycoplasma, fungal, and bacterial sterility was confirmed using a culture method. Cells were cultured in endothelial cell basal medium-2 (EBM-2; Lonza, Basel, Switzerland; CC3156) supplemented with microvascular endothelial SingleQuots kit (EGM-2MV; Lonza; CC4147). The medium was refreshed daily. The glucose concentration in normal glucose (NG) and HG medium was 5 and 30 mmol/L d-glucose, respectively. In cells cultured in NG, the concentration after 0, 8, 16, and 24 hours was 5.2, 4.7, 4.3, and 3.4 mmol/L, respectively.

Primary human glomerular microvascular endothelial cells were purchased from Cell Systems (ACBRI 128; Kirkland, WA). These cells were cultured the same as HRECs.

Primary human retinal pericyte cells (HRPCs) were purchased from Cell Systems (ACBRI 183). These cells were cultured in Dulbecco’s modified Eagle’s medium with t-glutamine and sodium bicarbonate, supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA; MT35010CV) and penicillin/streptomycin. The medium was refreshed daily in NG or HG conditions, same as for HRECs.

The 293T cells were purchased from ATCC (Manassas, VA). These cells were cultured in Dulbecco’s modified Eagle’s medium with t-glutamine and sodium bicarbonate, supplemented with 10% fetal bovine serum and penicillin/streptomycin in a 5% CO2 tissue culture incubator.

For lentivirus production, 70% confluent 293T cells were transfected with Lipofectamine 2000 (Invitrogen, Waltham, MA; catalog number 11668019) complexed with the packaging plasmid (psPAX2), envelope plasmid (pVSVg), and lentiviral plasmid of interest (mito-roGFP2-Orp1 or pHAGE-mtKeima (131626; Addgene, Watertown, MA)). The supernatant containing the virus was collected for 3 consecutive days, aliquoted, and stored at −80°C. Primary cells were infected with lentivirus harboring mito-roGFP2-ORP1 or pHAGE-mtKeima with 8 μg/mL polybrene reagent added to the medium. On the following day, the medium was replaced with complete growth medium. The infection efficiency was routinely >80% across all experiments.

Glucose Consumption Rate

NG-HRECs and HG-HRECs were plated into 24-well plates and cultured at full confluence in complete endothelial cell media (Lonza) containing 5 mmol/L (NG) or 30 mmol/L (HG) glucose. Medium was refreshed every 24 hours. The glucose level in the supernatant was measured every 8 hours (0, 8, 16, and 24 hours) using a glucometer with glucose test strips (Contour Next; CVS). The glucose values were extrapolated from the standard curve’s trendline equation. For the standard curve, we used Dulbecco’s modified Eagle’s medium (catalog number 103680-100; Agilent, CA) without glucose, supplemented with pyruvate, L-glutamine, and the bullet kit from Lonza to mimic the medium used for culturing HRECs. Multiple dilutions of glucose (0, 10, 20, 30, and 40 mmol/L) were used to plot the standard curve.

Assessing Cell Death

LDH

Lactate dehydrogenase (LDH), a measure of membrane integrity for cell-mediated cytotoxicity, was quantified using colorimetric CytoTox96 nonradioactive cytotoxicity assay (Promega, Madison, WI; G1780). LDH is a stable cytosolic enzyme that is released on cell lysis. The LDH activity that was released in the culture supernatant was measured with a coupled enzymatic assay following the manufacturer’s instructions. The OD was determined using a Synergy H1 spectrophotometer (Agilent). The amount of color formed is proportional to the number of lysed cells. For each experimental group, the amount of released LDH was normalized to the total LDH level, which was obtained by lysing cells using the lysis buffer supplied with the kit.

Fluorescence-Activated Cell Sorting

NG-HRECs and HG-HRECs were cultured in a 6-well tissue culture plate. For oxidative stress—induced death, confluent monolayers were treated with vehicle or 5 mmol/L TBH for 4 hours. Cells were collected by trypsinization and stained for annexin V and propidium iodine (Annexin VFITC Assay Kit; item number 600300; Cayman Chemical, Ann Arbor, MI), indicators of apoptosis and necrosis, respectively. Fluorescently stained cells were quantified on a Gallios cell sorting instrument (Beckman Coulter, Indianapolis, IN) with appropriate controls (singly stained and unstained cells). Cell death was defined as the sum of single- and double-positive cells.
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RNA Sequencing

The goal of this series of experiments was to assess the effect of HG on expression of genes in HRECs. To this end, triplicate dishes of confluent HRECs that were treated with either glucose condition for 1 or 10 days were harvested, and mRNA was isolated (RNasey isolation kit; Qiagen, Hilden, Germany; catalog number 74106) and submitted to the Bioinformatics Core at University of Illinois at Chicago. Two-way analysis of variance was applied to compare the changes in gene expression between 1-day NG/HG and 10-day NG/HG HRECs. For the 10-day data set, the list of genes with a statistically significant change in expression level was subjected to Gene Ontology analysis to identify inflammatory response genes. Inflammation-related genes were further filtered on the basis of the absolute number of counts, which was set as >1000.

PCR Analysis

Cells were lysed, and mRNA was isolated and used to synthesize cDNA (High-Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Waltham, MA; catalog number 4368813). Quantitative PCR was performed using Fast SYBR Green master mix (Applied Biosystems; catalog number 4385612) and run on the QuantStudio 7 Flex Real-Time PCR system (Applied Biosystems; catalog number 4485701). The CT of each transcript was normalized to the average CT of β-actin. Fold change was calculated using the 2-ΔCT method.

Western Blot Analysis

Confluent cultures of NG-HRECs and HG-HRECs were treated with tumor necrosis factor (TNF)-α (2.5 ng/mL) for the indicated time periods. After treatment, cells were rinsed with ice-cold phosphate-buffered saline (137 mmol/L NaCl, 2.7 mmol/L KCl, 8 mmol/L Na2HPO4, and 2 mmol/L KH2PO4) and lysed in 2× electrophoresis sample buffer (10 mmol/L EDTA; 2% SDS; 0.2 mol/L 2-mercaptoethanol; 20% glycerol; 200 mmol/L Tris-HCl, pH 6.8; and 0.2% bromophenol blue). Proteins were resolved on SDS-polyacrylamide gel and subjected to Western blot analysis.

The membrane was blotted for vascular cell adhesion molecule 1 (VCAM1; Abcam, Cambridge, UK; ab134047; rabbit monoclonal antibody (mAb); 1:1000 dilution) and intercellular adhesion molecule 1 (ICAM1; Abcam; ab109361; rabbit mAb; 1:1000 dilution) using primary antibodies. The protein level was normalized to β-actin (Cell Signaling; 8H10D10; mouse mAb; 1:1000 dilution). Secondary antibodies were used against mouse or rabbit (IRDye 800CW goat anti-rabbit or anti-mouse IgG; LiCor; 926-32211 or 926-32210, respectively), depending on the primary antibody source, to visualize the band. The blot images were acquired on a LiCor system (LI-COR, Lincoln, NE), and the relative amount of protein was quantified by ImageJ software version: 2.0.0-rc-69/1.52v (build 269a0ad53f; NIH, Bethesda, MD; https://imagej.net/software/fij). To measure the total level of mitochondria, we used antibodies TOM20 (Cell Signaling; D8T4N rabbit mAb; number 42406; 1:1000 dilution) and COXIV (Cell Signaling; 3E11; rabbit mAb; number 4850; 1:1000 dilution). Antibodies were validated using Western blot analysis of extracts from various cell lines, immunoprecipitation of the protein of interest, confocal immunofluorescence analysis of mouse tissues, and flow cytometric analysis of cell lines.

Seahorse Analysis

HRECs were plated at full confluence onto gelatin-coated XF96 well cell culture microplates (Agilent; catalog number 102416-100) in complete endothelial cell media from Lonza. The optimal cell confluence was determined using an ATP rate assay kit (Agilent; catalog number 103592-100). Before the start of oxygen consumption rate/ECCR measurements on the Seahorse XF96 machine (Agilent), the medium was replaced with Seahorse XF Dulbecco’s modified Eagle’s medium assay medium (Agilent; catalog number 103680-100) supplemented with 1.462 g/L glutamine, 110 mg/L pyruvate, and 5 or 30 mmol/L (for NG or HG, respectively, condition) d-glucose, which is equivalent to the culture conditions using complete Lonza media. Oxygen consumption rate was measured using MitoStress test (Agilent; catalog number 103015-100) following the manufacturer's protocol.

TBH Challenge Assay

The mito-roGFP2-Orp1sensor used in the TBH challenge assay was made from pLPCX mito-roGFP2-Orp1, which was a gift from Tobias Dick (64992; Addgene)23. The mito-roGFP2-ORP1 in the pLPCX plasmid was cut with Clal, blunted with T4DNA polymerase, and then cut with Bgl2. The resulting 1.5-kb DNA fragment was ligated into the Hpa1/BamH1-cut pLV-EF1a vector. Insert-containing constructs were detected on the basis of diagnostic BamH1/EcoR1 restriction fragments. Because the Clal site in the pLPCX mito-roGFP2-Orp1 plasmid is blocked by methylating the construct, this construct was propagated in dam-/dcm- bacteria.24,25 The mito-roGFP2-Orp1LVL-EF1a plasmid was used to make lentivirus (as described previously26), which was used to stably express mito-roGFP2-Orp1 in HRECs. The mito-roGFP2-Orp1 colocalized with mitochondrial proteins (data not shown), which is consistent with reports from other groups using this roGFP sensor in other cell types.25,26

Cells stably expressing mito-roGFP2-ORP1 were plated onto gelatin-coated black 96-well tissue culture plates. Each experimental condition was plated in triplicate, and three to four areas per well were selected for image acquisition. For time-lapse imaging, the medium was replaced with phenol red–free endothelial cell growth basal medium (Lonza; CC-
Measuring Mitophagy

The mitochondrially localized fluorescent protein Keima (MtKeima) was used to measure mitophagy in HRECs, human glomerular microvascular endothelial cells, and HRPCs. MtKeima has an excitation spectrum that changes according to pH.28 HRECs were transfected with lentivirus carrying pHAGE-mtKeima (131626; Addgene)29 using the procedure described above. HRECs stably expressing MtKeima were plated at full confluence onto gelatin-coated 35-mm glass-bottom dishes (MatTek; P35G-1.5-14-C). The next day, cells were transfected with siRNA targeting mitophagy regulators and then subjected to imaging. For live cell imaging on Zeiss confocal microscope, the medium was replaced to phenol red–free endothelial cell growth basal medium (Lonza; CC-3129) supplemented with microvascular endothelial SingleQuots kit (Lonza; EGM-2MV; CC4147) and 25 mmol/L HEPES. The 20× objective and Z-stack with six slices were used for image acquisition; at least five randomly selected sections of the dish were imaged for each experimental condition. Images were then converted into maximal intensity projection for further analysis on ImageJ software. Mitophagy was quantified by dividing red pixels to green pixels using Colocalization Threshold Analysis (Rcol) on ImageJ software.

HRECs stably expressing mitochondrially localized mito-roGFP2-Grx130 were stained for 30 minutes with lysosomal marker LysoTracker-red (Invitrogen; L7528) following manufacturer’s protocol. Medium was then replaced to phenol red–free endothelial cell medium supplemented with 25 mmol/L HEPES. The immunofluorescence images were acquired and processed as described above. Colocalization between red lysosomes and green mitochondria was quantified using Colocalization Threshold Analysis (Rcol) on ImageJ software, same as for MtKeima.

Urolithin A was purchased from Sigma (St. Louis, MO; SML1791).

siRNA

Confluent HRECs, plated onto a 6-well tissue culture plate, were transfected with siRNA in an antibiotic-free complete endothelial cell medium (Lonza). To this end, 10 mmol/L of ON-TARGETplus Human siRNA SMARTpool (Horizon Discovery, Waterford, UK) targeting MFN2 (L-012961-00), DNML (L-012092-00), OPTN (L-016269-00), OPA1 (L-005273-00), or nontargeting pool (Scr; D-001810-10-05) was complexed with DharmaFECT 1 transfection reagent (Horizon Discovery; catalog identifier T-2001) at 1:2 ratio in reduced serum Opti-MEM medium (Gibco; 31985070) and added to the medium. The next day, transfected cells were trypsinized and plated into a 96-well tissue culture plate (for TBH-challenge assay or LDH assay) or 8-well chamber slide (for transendothelial electrical resistance assay) in complete endothelial cell media. At 48-hour time point after transfection, transfected cells were subjected to the designated experimental assay. The extent of silencing was determined on both the protein and mRNA level using Western blot analysis and RT-qPCR, respectively, methods described above. The sequences of the primers used for RT-qPCR are listed in Table 1. Antibodies used for Western blot analysis were as follows: MFN2 (D2D10; rabbit mAb; number 9482; Cell Signaling), DNML1 (DR1P; D6C7; rabbit mAb; number 8570; Cell Signaling), OPA1 (D6U6N; rabbit mAb; number 80471; Cell Signaling), and OPTN (E4P8C; rabbit mAb; number 70928; Cell Signaling).

Barrier Function

Cell permeability was assessed by measuring changes in transendothelial electrical resistance using an electrical cell-substrate impedance sensing ZThera instrument (Applied Biophysics, Troy, NY), which was housed in a standard tissue culture incubator that was maintained at 37°C and 5%
CO₂. Briefly, transfected cells were plated into an 8-well chamber at full confluency. The following day, the medium was refreshed before the transendothelial electrical resistance measurements. Basal resistance was established within 2 hours, whereupon cells were stimulated with 2 nmol/L vascular endothelial growth factor or vehicle (phosphate-buffered saline) and continued to be monitored for up to 48 hours, as previously described.²⁴,³¹

## Results

### The Duration of Exposure to Hyperglycemia Influenced Susceptibility to Damage that Resulted from DM-Related Insults

We considered the effect of varying the duration of HG on several parameters that are associated with DR pathogenesis (inflammation and cell death). These experiments were done with HRECs cultured in either 5 mmol/L (90 mg/dL) or 30 mmol/L (540 mg/dL) d-glucose. Many groups have reported that exposure of endothelial cells to inflammatory cytokines increases expression of ICAM1 and VCAM1 and thereby promotes extravasation of immune cells, which is a key step in inflammation.³² Stimulation of HRECs with TNF-α increased the expression of ICAM1 and VCAM1, and the extent of expression was comparable in cells treated with HG for either 6 hours or 1 day (Figure 1A and Supplemental Figure S1). Prolonging the exposure to HG reduced the magnitude of TNF-α-induced expression of ICAM1 (10 days) and VCAM1 (3 and 10 days) (Figure 1A). These results indicate that extended exposure of cells to HG attenuated TNF-α-induced activation.

We also investigated the effect of HG on steady-state expression of inflammation-related genes. RNA-sequencing analysis indicated that expression of 55 genes underwent a statistically significant change after 10 days of exposure to HG (Supplemental Figure S2). In contrast, the expression of only four of these genes changed after 1 day of HG

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**Figure 1** The duration of exposure to high glucose (HG) influenced susceptibility to damage caused by diabetes mellitus--related insults. A: Human retinal endothelial cells (HRECs) that had been incubated in either normal glucose (NG) or HG for the indicated duration were subsequently exposed to vehicle (veh) or TNF-α. The amount of vascular cell adhesion molecule 1 (VCAM1) and intercellular adhesion molecule 1 (ICAM1) signal was normalized to the β-actin signal. The resulting data were expressed as a percentage of the response in NG cells. Each experiment included three sample repeats. Statistical significance was assessed by two-tailed t-test with unpaired two-sample equal variance. B: HRECs were cultured in media containing either NG or HG for the indicated duration. Top panel: To measure basal cell death, lactate dehydrogenase (LDH) was allowed to accumulate in the conditioned medium, which was harvested, and LDH activity was assessed as described in Materials and Methods. To determine death in response to oxidative stress, the medium was replaced and then vehicle or TBH (final concentration of 5 mmol/L) was added for 4 hours, whereupon the LDH activity in the medium was quantified. All experiments were repeated at least three times. The bar graph shows a single representative experiment in which each experimental condition was assayed in triplicate. Statistical significance was assessed by two-tailed t-test with unpaired two-sample equal variance. Bottom panel: HRECs that had been incubated in either NG or HG for the indicated duration were either left resting or treated with vehicle or 5 mmol/L TBH for 4 hours. The LDH activity was measured as described above. C: Same as B, except death was assessed by fluorescence-activated cell sorting (FACS) analysis of cells stained with annexin V and propidium iodide (PI). Cell death was quantified as the sum (three quadrants in Supplemental Figures S3 and S4) of single-positive (annexin V or PI) and double-positive (annexin V and PI) cells. Statistical significance was assessed by two-tailed t-test with unpaired two-sample equal variance. Data are expressed as the average ± SD (A and C). *P < 0.05.
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(Supplemental Figure S2). We conclude that the duration of exposure to HG affects both the steady-state expression of inflammation-related genes and responsiveness to cytokine-induced activation. The observation that prolonging the exposure to HG reduced TNF-α–driven activation was counter to the dogmatic expectation that HG causes progressive damage.

We performed the following experiments to test if the duration of exposure to HG influenced basal- or insulin-induced death. As shown in Figure 1B, we observed that after initially increasing (after 3 days of HG), basal cell death was no longer elevated after 10 days of HG. Similarly, oxidative stress–induced cell death was higher in cells that had been cultured in HG for 1 day, and then became lower after 10 days of HG (Figure 1B). In these experiments, cell death was assessed by measuring release of LDH. Evaluating cell death by expression of annexin V and permeability to propidium iodine confirmed the key observation that extended exposure to HG made cells resistant to cell death (Figure 1C and Supplemental Figures S3 and S4).

These observations resonate with a previous report that HG induced a slight HRECs’ death at 4 and 6 days of treatment, and prolonging the exposure for 4 weeks no longer activated cell death.33

Although Figure 1B shows that cell death increased after 3 days of HG, we did not detect a decline in the total protein (Figure 1A). More important, LDH data were normalized for cell number by expressing the results as a fraction of the total LDH. Furthermore, in the course of daily maintenance of the cells, we noticed that the cell monolayer was invariably confluent, and that the small number of floating cells did not differ across experimental conditions (data not shown). A plausible explanation for our inability to detect a decline in the overall cell number when cell death increased is the fact that cells were continuously cultured in 5% serum-containing medium, which supports proliferation. Consequently, cell proliferation likely replaced cells that died, and hence maintained a comparable number of cells across experimental conditions.

Taken together, these findings indicate that prolonged exposure of HRECs to HG made them resilient to the deleterious effects of HG instead of making them more vulnerable. Such adaptation was most apparent when cells were challenged, and involved resistance to cytokine-induced activation and oxidative stress–driven cell death.

Adaptation Was Associated with Improved Mitochondrial Functionality

We focused on the mitochondria to further characterize adaptation. Existing publications demonstrating that mitochondria adapt to environmental changes16,18 motivated us to consider if the duration of exposure to HG influenced mitochondrial functionality. Seahorse analysis revealed that HG was initially detrimental; it reduced basal oxygen consumption (Figure 2A), the spare respiratory capacity (Figure 2B), and ATP production (Figure 2C). All of these parameters improved (instead of further declining) as the duration of exposure to HG was prolonged (Figure 2).

We also investigated the effect of HG on mitochondrial functionality using a newly developed approach that evaluated the mitochondria’s ability to resolve acute oxidative stress. This technique uses an roGFP sensor, which has the following four features. i) The green fluorescent protein (GFP) portion of the sensor is mutated so that its fluorescence reflects oxidative status. ii) It is tagged with a mitochondrial localization signal. iii) It is fused with the yeast peroxidase ORP1 to impart preference for hydrogen peroxide. iv) It is reversible and, therefore, can be used to observe both an increase, and subsequent decrease, in

**Figure 2** Exposure to high glucose (HG) initially compromised the oxygen consumption rate (OCR), and then improved it. Human retinal endothelial cells that had been incubated in either normal glucose (NG) or HG for the indicated duration were subsequently assayed for oxygen consumption rate, as described in Materials and Methods. A: Basal respiration was calculated after subtracting nonmitochondrial respiration. B: Spare respiratory capacity, which indicates the capability of the cell to respond to an energetic demand, was calculated on the basis of the difference between the basal respiration and maximal respiration. C: ATP production shows ATP produced by the mitochondria that contribute to meeting the energetic needs of the cell. The asterisks indicate statistical significance, which was assessed by two-tailed t-test with unpaired two-sample equal variance. The data shown in this figure are from a single representative experiment in which 10 to 12 wells were used for each experimental condition. Similar results were obtained in three independent experiments. *P < 0.05.
oxidative stress in live cells and in real time. Figure 3A shows images of HRECs stably expressing the mito-roGFP2-Orp1 sensor, and the oxidation-dependent change in signal intensity. Such roGFP sensors have been characterized extensively and used to evaluate changes in oxidative stress in a variety of experimental conditions.\textsuperscript{34–36}

We stably expressed this mito-roGFP2-Orp1 sensor in HRECs, and exposed the resulting cells to TBH and monitored the change (increase and resolution) of mitochondrial oxidative stress for at least 5 hours. TBH caused an acute and transient increase in mitochondrial hydrogen peroxide; the resolution took longer at higher doses of TBH (Figure 3B). These experimental conditions (dose and duration of exposure to TBH) had no effect on cell viability (data not shown). This TBH challenge assay enabled us to assess the capacity of the mitochondria to resolve oxidative stress. Unless indicated otherwise, the assay was performed using 270 \( \mu \text{mol/L} \) TBH.

We used the TBH challenge assay to determine the effect of different durations of exposure to HG on mitochondrial functionality. The performance in the TBH challenge assay was unaffected by exposure to HG for 0.5 days; the response curves of cells cultured in NG and HG overlapped (Figure 3C). After 1 day of HG, the TBH-induced increase in oxidative stress was higher and persisted longer in HG versus NG cells (Figure 3C). Further increasing the duration of the exposure to HG reversed this effect. After 10 days, the response of HG cells improved relative to NG cells; TBH induced a smaller increase and faster resolution of oxidative stress (Figure 3C). The TBH challenge assay results confirm the conclusions from the Seahorse analysis that exposure to HG initially compromised mitochondrial functionality, which improved (instead of declining further) as the duration of exposure was prolonged. These mechanistic insights further support the novel concept that HRECs adapt to HG in ways that improve their ability to withstand its deleterious effect.

Mitochondrial oxidative stress did not increase even after 10 days of exposure to HG; the basal level of H2O2 (before addition of TBH) was comparable in NG and HG cells (Figure 3C). Other reactive oxygen species, measured with additional roGFPs or fluorescent oxidation-reduction-sensitive dyes, were also unchanged by HG (data not shown). The mitochondria’s resistance to HG-induced...
Mitophagy was elevated in adapted cells. A: MtKeima-expressing human retinal endothelial cells (HRECs) were cultured in normal glucose (NG) or high glucose (HG) for 10 days and visualized using confocal microscopy. Representative confocal images show the overlay of red and green fluorescence. The positive control for these studies were NG cells treated with FCCP (10 µmol/L), which is a mitochondrial membrane protonophore. The red signals (white arrows) point to examples of mitochondria that are within an acidic compartment, such as the lysosome. B: The extent of mitophagy in MtKeima-expressing HRECs subjected to the indicated experimental conditions was quantified as described in Materials and Methods. The data presented are from at least three independent experiments, which were normalized to the level of mitophagy in NG-HRECs. Statistical significance was calculated by two-tailed t-test with unpaired two-sample equal variance. C: HRECs that were cultured in NG or HG for 10 days were harvested, and RNA was isolated and subjected to RT-qPCR using primers for the indicated genes. To calculate the relative amount of mRNA, the Ct of each transcript was normalized to the average Ct of β-actin. Fold change (y axis) was calculated using the 2⁻ΔΔCt method. The P value was determined using two-tailed t-test with unpaired two-sample equal variance. Similar results were obtained on at least three independent occasions. D: HRECs were treated with NG or HG for 10 days, harvested, and lysed. Proteins were resolved on an SDS-polyacrylamide gel and subjected to Western blot analysis (WB) using the indicated antibodies. The signal was quantified using ImageJ software; COX4 and TOMM20 were normalized to β-actin. Each experiment included triplicate samples. Data indicate the average ± SD of triplicates of a single representative experiment (C) or the average ± SD of three independent experiments (D); n = 3 (C). *P < 0.05. Scale bar = 20 µm (A).
the high degree of heterogeneity of the mitochondrial morphology among cells in either glucose condition precluded use of mitochondrial morphology as a parameter to assess mitophagy (Supplemental Figure S7). In contrast, the quantitative approaches measuring the degree of mitophagy of the entire population of cells showed that HG elevated mitophagy (Figure 4 and Supplemental Figures S5 and S6).

Mitochondrial Dynamics Were Involved with Maintenance of HIMA

To assess the role of mitochondrial dynamics in maintenance of hyperglycemia-induced mitochondrial adaptation (HIMA), we suppressed expression of four genes that are involved in various aspects of this quality control system: fission (DNM1L), fusion (OPA1 and MFN2), and mitophagy (OPTN and MFN2). HIMA was compromised (reduced performance in the TBH challenge assay) by suppression of any one of these four genes (Table 2, Figure 5, and Supplemental Figure S8). Furthermore, reducing expression of fission genes suppressed mitophagy (Supplemental Figure S8), which demonstrates the interrelatedness of the various component of mitochondrial dynamics. The effect on HIMA was greatest in MFN2 knockdown cells (Figure 5 and Table 2), perhaps because MFN2 is required for multiple components of mitochondrial dynamics.20 We conclude that maintenance of HIMA required enhanced mitochondrial dynamics.

HG-Induced Adaptation Was Beneficial

We proceeded to investigate if loss of HIMA would compromise the cells’ ability to endure HG. To this end, we suppressed MFN2 expression (Figure 5) and assessed the impact on susceptibility to insult-induced death and functionality of the cells. Loss of HIMA increased vulnerability to both basal- (Figure 5D) and oxidative stress—induced cell death (Figure 6A). Furthermore, barrier function was compromised (ie, increased the permeability of confluent monolayers) (Figure 6B). Although the increase in basal cell death may contribute to enhanced permeability of MFN2 knockdown cells, reducing the level of DNM1L reduced barrier function without influencing cell death (Supplemental Figure S8). Finally, MFN2 knockdown cells lost their ability to relax the barrier further in response to vascular endothelial growth factor (Figure 6B). Taken together, our results indicate that HRECs adapted to HG and thereby acquired protection from HG-induced harm.

The recent discovery that MFN2 contributes to barrier function39 raises the possibility that the enhanced permeability of MFN2 knockout cells is not only caused by dysfunctional mitochondrial dynamics. However, barrier function was also compromised when DNM1L (required for fission) expression was suppressed (Supplemental Figure S8E).

Although suppressing mitochondrial dynamics eliminated certain features of HIMA, others persisted. Cells remained resistant to TNF-a—induced activation, which we monitored by ICAM1 and VCAM1 expression, as in Figure 1 (Figure 6B). These findings suggest that although elevated mitochondrial dynamics are an essential component of HIMA, they are not the only one.

In an attempt to eliminate HIMA in HRECs by an alternative approach, we prolonged the exposure to HG for up to 15 days, which approached the passage limit of these primary cells. Prolonged exposure to HG did not eliminate HIMA (data not shown), suggesting that this is a relatively stable state.

Urolithin A Promoted Partial Adaptation of NG Cells

We also tested if increasing mitophagy in NG cells would suffice to induce adaptation. Exposure of NG cells to urolithin A, an agent that enhances mitophagy and improves mitochondrial health,34 increased mitophagy and performance in the TBH challenge assay (Figure 7, A and B). However, it did not attenuate TNF-a—induced activation or reduce basal- or insult-induced death (Figure 7, C and D, and Supplemental Figure S9). Incomplete adaptation was also observed with other mitophagy-promoting agents (Torin2, mTor inhibitor; and SR3677, Rho kinase inhibitor; data not shown). We conclude that although enhanced mitophagy is essential for cells to maintain key features of HIMA, it is not sufficient to acquire it.

The Effect of Osmolality

We tested the effect of osmolality by comparing the behavior of cells cultured for 10 days in 5 mmol/L D-glucose, 30 mmol/L D-glucose, or 5 mmol/L L-glucose (present in the culture medium) + 25 mmol/L L-glucose (LG) (Supplemental Figure S10). The results show that 10

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### Table 2: Importance of Mitochondrial Dynamics for Maintenance of HIMA

<table>
<thead>
<tr>
<th>Variable</th>
<th>DNML</th>
<th>OPA1</th>
<th>OPTN</th>
<th>MFN2</th>
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</thead>
<tbody>
<tr>
<td>Role in mitochondrial dynamics</td>
<td>Fission</td>
<td>Fusion</td>
<td>Mitophagy</td>
<td>Mitophagy; fusion</td>
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<td>Extent of siRNA-mediated knockdown (mRNA/protein), %</td>
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<td>Compromised performance in the TBH challenge assay</td>
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<td>Y/N/Y/Y</td>
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<tr>
<td>Suppression of mitophagy</td>
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<td>Y/N/Y</td>
<td>Y/N/Y/N</td>
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</tr>
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HIMA, hyperglycemia-induced mitochondrial adaptation; N, no; Y, yes.

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days of treatment with LG did not induce basal cell death, but promoted resistance to oxidative stress–induced cell death as in HG. The resistance to TBH-induced death was not due to increased mitophagy; moreover, LG had no effect on mitochondrias’ respiratory capacity.

The Response of Other Cell Types to Hyperglycemia

It has been reported that Muller and human retinal pigment epithelium cells respond to HG by up-regulating the level of oxidative stress and cytokine production. To investigate if pericytes, the other cell type in retinal capillaries, also underwent adaptation, we subjected them to a similar series of experiments as was done with HRECs. Prolonged exposure of primary HRPCs to HG resulted in resistance to HG-induced death (Figure 8A) but not to oxidative stress–induced death (Figure 8B). Furthermore, the underlying mechanism by which HRPCs adapted was not the same as for HRECs; there was no improvement in the TBH challenge assay, and mitophagy was not increased (Figure 8, C and D). We conclude that both vascular cell types of retinal capillaries adapt to HG, but the degree of protection and process by which they achieve this state are not the same.

In contrast to HRECs, primary human glomerular microvascular endothelial cells did not adapt to HG (Figure 8, E and F). HG promoted death of unchallenged cells and did not protect from oxidative stress–induced death. Furthermore, there was no improvement of performance in the TBH challenge assay or change in the level of mitophagy. Thus, HIMA is not a universal feature of endothelial cells, even among those that reside in organs that undergo DM-induced dysfunction after a considerable delay.

Discussion

We report that prolonged exposure of primary human retinal vascular cells to HG decreases (instead of increases) their vulnerability to the deleterious effects of HG. The underlying mechanism in endothelial cells involves enhanced mitochondrial dynamics and mitophagy, although elevating mitophagy in normal glucose cells was insufficient to induce this state. Although HRPCs also undergo adaptation in response to HG, the degree of protection from death was attenuated and the underlying mechanism appears to be distinct from HIMA.
Hyperglycemia Triggers an Endogenous System that Limits HG-Induced Damage

Figure 6  Loss of hyperglycemia-induced mitochondrial adaptation compromised the viability and functionality of high glucose (HG) human retinal endothelial cells (HRECs). A: HRECs that were cultured in HG for 10 days were mock transfected [control (Cntr)] with either scrambled siRNA (siScr) or MFN2-targeting siRNA (siMFN2). At 48 hours after transfection, the medium was refreshed, and cells were treated with vehicle (Veh) or 5 mmol/L TBH for 4 hours and then subjected to the lactate dehydrogenase (LDH) activity assay. The P value indicates a statistically significant difference between the two groups. A representative experiment is shown in Figure 6A; similar results were observed on three independent occasions. B: HRECs that were cultured in HG for 10 days were transfected with either siScr or siMFN2. At 48 hours after transfection, the cells were treated with 2.5 ng/mL tumor necrosis factor (TNF)-α for 6 hours, lysed, and subjected to Western blot analysis, as in Figure 1. The experiment was repeated four times on independent occasions. A representative experiment is shown. C: The transendothelial electrical resistance (TEER) of cells treated as described in A was assessed (as described in Materials and Methods) under either unstimulated (left panel) or vascular endothelial growth factor (VEGF)—stimulated conditions (right panel). The experiment was repeated at least three times; representative TEER tracings are shown. *P < 0.05. ICAM1, intercellular adhesion molecule 1; VCAM1, vascular cell adhesion molecule 1.

Preconditioning, a brief exposure to a sub-threshold level of injury, protects organs, such as the brain and heart, from subsequent insult. The underlying mechanism of this phenomenon involves changes in the mitochondria, which include elimination of dysfunctional mitochondrial (by mitophagy).40,41 More recent studies have elucidated the molecular mediators and relevance to additional organs. For instance, the exercise capacity of skeletal muscle is dependent on autophagy to clear dysfunctional mitochondria.44 Similarly, 1 week of DM protected mice from ischemia-induced injury of skeletal muscle.44 Similarly, 1 week of DM protected mice from ischemia/reperfusion-induced heart injury compared with non-DM mice.45 The underlying mechanism of this phenomenon has not been addressed. Compared with ischemia- or hypoxia-induced preconditioning, HG-induced preconditioning is a largely unexplored area of research.

In contrast to the extensive literature focused on preconditioning in cardiovascular disease, there are only a handful of publications investigating DM/HG and the concept of preconditioning. Patients with type I DM are protected from ischemia-induced injury of skeletal muscle.44 Similarly, 1 week of DM protected mice from ischemia/reperfusion-induced heart injury compared with non-DM mice.45 The underlying mechanism of this phenomenon has not been addressed. Compared with ischemia- or hypoxia-induced preconditioning, HG-induced preconditioning is a largely unexplored area of research. There is compelling evidence that mitochondrial dysfunction is associated with pathogenesis of DR. Single-nucleotide polymorphisms in genes that regulate mitochondrial functionality (UPC1 and UPC2) are associated with diabetic retinopathy in patients.46,47 In early DR, the mitochondrial content declines in the retina of mice and...
Figure 7 Urolithin A (UA) induced partial hyperglycemia-induced mitochondrial adaptation. A: MtKeima-expressing normal glucose (NG) human retinal endothelial cells (HRECs) were treated with vehicle or 1 μmol/L UA for 3 days, and then the level of mitophagy was assessed, as described in Figure 6. The experiment was repeated at least three times, and the statistical significance (P < 0.05) for a single time point is indicated by asterisks. B: mito-roGFP2-Orp1—expressing NG HRECs were treated with vehicle or UA (1 μmol/L) for 3 days and then subjected to the TBH challenge, as described in Figure 3. Similar results were observed in at least three independent experiments. The statistical significance for a single time point is indicated with an asterisk. A representative experiment is shown. C: NG HRECs were treated with vehicle or UA (1 μmol/L) for 3 days and then stimulated with 2.5 ng/mL tumor necrosis factor (TNF)-α for 6 hours. The cells were lysed and subjected to Western blot analysis using the indicated antibodies. The bar graph is the compilation of three independent experiments, which were processed as described in Figure 1. D: NG HRECs were treated with vehicle or UA (1 μmol/L) for 3 days and then both basal- and TBH-induced cell death was assayed, as described in Figure 1. Similar results were obtained on at least three independent occasions. Data are expressed as the average ± SD for three independent experiments, normalized to control (Cntr; A), or the average ± SD of triplicates of a single representative experiment (D). *P < 0.05. ICAM1, intercellular adhesion molecule 1; VCAM1, vascular cell adhesion molecule 1.

human donor eyes. As the disease progresses, mitophagy is impaired and dysfunctional mitochondria accumulate.48,49 Similarly, damaged mitochondria accumulate as the homeostasis between biogenesis and turnover of mitochondria is compromised by aging.50 Furthermore, mitochondrial disintegration/fragmentation activates NLRP3-mediated production of inflammatory cytokines and necroptosis.51 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. Attempts to prevent diabetic retinopathy with mitochondria-targeted therapy are beneficial in some, but not all, cases.49,52–54 Thus, DM-induced damage of the mitochondria within the retina, which triggers a self-amplifying loop of dysfunction, appears to be a driver of DR pathogenesis.

The compelling role of mitochondrial dysfunction in the development of DR supports this report’s central concepts that cells harbor a system to protect from DM-induced damage, and that this system’s purpose is to preserve the functionality of the mitochondria (Figure 9). Exposure to HG initially compromises the health of the mitochondria, which is detectable by decreased performance in the TBH challenge assay (Figure 3C). A subsequent increase in mitochondrial dynamics is at least one of the drivers of improved mitochondrial health observed after 10 days of HG. Finally, we posit that loss of HIMA sets the stage for advancing to DR (Figure 9).

Although the discovery of HIMA is an important first step, the work described herein does not provide a comprehensive description of this process. Open questions include whether mitochondria possess a trigger(s) that initiates and commits cells to HIMA? It may be a multistep process instead of a single event that occurs at a precise time point; we observe distinct changes at different times (attenuation of TNF-α–induced expression of VCAM1 at 3 days and elevation of mitophagy at 7 days). Elucidating the full spectrum of HIMA-associated changes and their functional relationships will enable progress in this arena.

In addition to HRECs, it appears that the prolonged exposure of primary human retinal pericytes to HG also increases their ability to resist HG-induced death, although...
by a different mechanism. In contrast, primary human glomerular endothelial cells did not adapt under our experimental conditions. We chose this additional endothelial cell type because like DR, diabetic nephropathy does not develop quickly following the onset of DM. If an endogenous system to protect the kidney from DM-driven dysfunction exists, then it may not reside within the glomerular endothelium.

Figure 8  Hyperglycemia-induced mitochondrial adaptation did not occur in all cell types. A and E: Primary human retinal pericyte cells (HRPCs) and human glomerular microvascular endothelial cells (HGECs) were cultured in either normal glucose (NG) or high glucose (HG) for the indicated duration and then cell death was quantified as described in Figure 5. B and F: Same as described in the legend of Figure 1. C and G: mito-roGFP2-Orp—expressing HRPCs and HGECs were cultured in either NG or HG for 10 days and then subjected to the TBH challenge assay, as described in Figure 3. No statistically significant differences were observed between NG and HG cells in three independent experiments. D and H: MtKeima-expressing HRPCs and HGECs were cultured in either NG or HG for 10 days and then the level of mitophagy was quantified, as described in Figure 4. No statistically significant differences were observed between NG and HG cells in three independent experiments. Data are expressed as the average ± SD (A and E). n = 3 (A and E). *P < 0.05. FACS, fluorescence-activated cell sorting.

Figure 9  Overview of the discoveries and their potential clinical relevance. Culturing human retinal endothelial cells in high glucose (HG) initially compromised their mitochondria. The cells respond by adapting, which includes clearance of the dysfunctional mitochondria via mitophagy. Such adaptation is a plausible contributor to the underlying mechanism responsible for the long delay between the onset of diabetes and manifestation of diabetic retinopathy. Furthermore, loss of adaptation may be a prerequisite for development of retinopathy in patients with diabetes.
An inherent component of the HIMA concept is that improving the functionality of a subset of retinal cells will be beneficial for the entire retina. Previous publications report that even a small reduction in the degree/type of insult that the retina experiences is sufficient to protect from developing retinopathy in animals that have DM. Suppressing the inflammatory process within myeloid cells,\textsuperscript{57–59} altering phototransduction or visual cycle within photoreceptor cells/retinal pigment epithelial cells,\textsuperscript{60,64} or overexpressing metallothionein in endothelial cells\textsuperscript{62} attenuates or completely prevents animals with DM from succumbing to DR. Together, these discoveries suggest that manifestation of DR involves a relatively small shift in the balance between endogenous systems that prevent DM-driven damage and drivers of pathogenesis.

Does HIMA exist in vivo, does it protect patients from DR, and is its demise a prerequisite for progression to DR? Our ongoing research is focused on addressing these open questions.

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Supplemental Data

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References

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31. Li Y, Yan Z, Chaudhry K, Kazlauskas A: The renin-angiotensin-aldosterone system (RAAS) is one of the effectors by which vascular endothelial growth factor (VEGF)/anti-VEGF controls the endothelial cell barrier. Am J Pathol 2020, 190:1971–1981
57. Randow F, Youle RJ: Spatiotemporal control of ULK1 activation by depending activation of mitophagy. Cells 2019, 8:213