High Glucose Induces Mitochondrial Dysfunction in Retinal Müller Cells: Implications for Diabetic Retinopathy

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PURPOSE. To investigate whether high glucose (HG) induces mitochondrial dysfunction and promotes apoptosis in retinal Müller cells.

METHODS. Rat retinal Müller cells (rMC-1) grown in normal (N) or HG (30 mM glucose) medium for 7 days were subjected to MitoTracker Red staining to identify the mitochondrial network. Digital images of mitochondria were captured in live cells under confocal microscopy and analyzed for mitochondrial morphology changes based on form factor (FF) and aspect ratio (AR) values. Mitochondrial metabolic function was assessed by measuring oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) using a bioenergetic analyzer. Cells undergoing apoptosis were identified by differential dye staining and TUNEL assay, and cytochrome c levels were assessed by Western blot analysis.

RESULTS. Cells grown in HG exhibited significantly increased mitochondrial fragmentation compared to those grown in N medium (FF = 1.7 ± 0.1 vs. 2.3 ± 0.1; AR = 2.1 ± 0.1 vs. 2.5 ± 0.2; P < 0.01). OCR and ECAR were significantly reduced in cells grown in HG medium compared to those grown in N medium (steady state: 75% ± 20% of control, P < 0.02; 64% ± 22% of control, P < 0.02, respectively). These cells also exhibited a significant increase (~2-fold) in the number of apoptotic cells compared to those grown in N medium (P < 0.01), with a concomitant increase in cytochrome c levels (247% ± 94% of control, P < 0.05).

CONCLUSIONS. Findings indicate that HG-induced mitochondrial morphology changes and subsequent mitochondrial dysfunction may contribute to retinal Müller cell loss associated with diabetic retinopathy.

Keywords: high glucose, diabetes, diabetic retinopathy, retinal Müller cells, mitochondria
the context of Müller cell loss. As documented in our previous studies, HG induces mitochondrial fragmentation and thereby compromises mitochondrial functionality in retinal vascular cells.6,7 Additionally, HG has been shown to induce mitochondrial morphology changes in various other cell types such as rat cardiomyoblasts23 and human mesangial cells.24 The purpose of the current study is to establish whether Müller cell loss is attributable to mitochondrial dysfunction by investigating the effects of HG on mitochondrial morphology; mitochondrial membrane potential heterogeneity; oxygen consumption rate (OCR); and extracellular acidification rate (ECAR) concomitant with cytochrome c release.

**METHODS**

**Cell Culture**

We grew rMC-1 on poly-D-lysine coated, glass-bottom culture dishes (MatTek Corp., Ashland, MA, USA) in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Sigma-Aldrich Corp., St. Louis, MO, USA); antibiotics; and antimycotics. To determine the effect of HG on mitochondrial morphology, rMC-1 were grown for 7 days under normal (5 mmol/L) or HG (30 mmol/L) conditions. In parallel, rMC-1 were grown in mannitol (30 mmol/L) for osmotic control. After 7 days of HG exposure, cells were subjected to mitochondrial staining and examined through live cell imaging using confocal microscopy.

**Fluorescent Probes**

To determine the effect of HG on mitochondrial morphology, rMC-1 grown in N or HG medium for 7 days were incubated at 37°C in a 5% CO2 incubator with 30 nM membrane potential-dependent dye (MitoTracker Red [MTR] CMXRos; Invitrogen-Molecular Probes, Eugene, OR, USA) for 15 minutes before imaging and kept in medium during the imaging processes.

**Confocal Microscopy**

Live cell imaging of mitochondria was accomplished using a confocal microscope (Zeiss LSM 710-Live Duo; Carl Zeiss, Oberkochen, Germany) with a ×63 oil-immersion objective. Throughout the process, cells were incubated at 37°C in a 5% CO2 humidified microscope stage chamber. We subjected MTR to 543-nm helium/neon laser excitation and emission was recorded through a band-pass 650–710-nm filter. Fields were randomly selected, and those with similar cell densities were then imaged. To observe individual mitochondria, z-stack images were taken in a series of 10 to 15 slices per cell with a thickness of 0.5 μm per slice.

**Mitochondrial Morphology Analysis**

Mitochondrial morphology was quantified using a computer-assisted morphometric analysis application to calculate form factor (FF) and aspect ratio (AR) values. Images of mitochondria were analyzed using Image J software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) by first processing with a median filter to obtain isolated and equalized fluorescent pixels. Mitochondria were subjected to particle analysis to obtain FF values (4π × area/perimeter2) and AR values (ratio of the lengths of major and minor axes). Aspect ratio is a measure of mitochondrial length; a value of 1 indicates a perfect circle and increases with elongated and elliptical mitochondria. Form factor is an indication of both mitochondrial length and degree of mitochondrial branching. A form factor value of 1 reflects a circular, unbranched mitochondrion, while a higher FF value indicates a longer, more branched mitochondrion.

**Cellular Oxygen Consumption and Extracellular Acidification**

Oxygen consumption and extracellular acidification rates of rMC-1 were measured by a bioenergetic assay (XF 24; Seahorse Bioscience, Billerica, MA, USA). We plated and cultured rMC-1 in a 24-well microplate (Seahorse Bioscience) for 7 days in N or HG medium to assess cellular oxygen consumption and extracellular acidification rates. For analysis, growth medium was replaced with nonbuffered medium (XF Assay; Seahorse Bioscience) and cells were incubated at 37°C in a non-CO2 incubator for 60 minutes to allow the temperature and pH to reach equilibrium. The bioenergetics assay (Seahorse Bioscience) was used to measure extracellular flux changes in oxygen and protons in the media immediately surrounding the cells. After steady-state oxygen consumption and extracellular acidification rates were obtained, ATP synthase inhibitor oligomycin (5 μM) and the proton ionophore FCCP (carbonyl cyanide-4-[trifluoromethoxy] phenylhydrazone; 1 μM), a mitochondrial uncoupler, were injected sequentially through reagent delivery chambers to each well in the microplate to obtain the maximum oxygen consumption rate. Finally, a mixture of 5 μM rotenone (a mitochondrial complex I inhibitor) and 5 μM antimycin A (an electron transport blocker) was injected to verify that observed changes were due mainly to mitochondrial respiration.

**Mitochondrial Membrane Potential Heterogeneity Analysis**

To examine mitochondrial membrane potential heterogeneity in rMC-1, a ratiometric image-analysis approach was applied based on dual staining, as previously described.5 Tetramethylrhodamine ethyl ester (TMRM) is a quickly equilibrating membrane potential–dependent dye that does not inhibit mitochondrial respiration. MitoTracker Green (MTG; Thermo Fisher Scientific, Waltham, MA, USA) is a membrane potential–independent dye. Using the product ratio of TMR to MTG dyes maintains the voltage dependency of TMR. In addition, this ratio is independent of fluorescence intensity variability from one focal plane to another.25 Images were acquired using confocal microscopy and individual mitochondria were analyzed. Mitochondrial potential heterogeneity was determined by the deviation in the ratio of TMRM (red; excitation/emission: 550 nm/574 nm) to MTG (green; excitation/emission: 490 nm/516 nm) fluorescence intensity for several mitochondria within each cell and was quantified using analytical software (Metamorph; Molecular Devices, Sunnyvale, CA, USA).

**Cytochrome C Release**

We washed rMC-1 grown in normal and HG with PBS and lysed with 0.1% Triton X-100 buffer containing 10 mmol/L Tris, pH 7.5, 1 mmol/L EDTA, and 1 mmol/L phenylmethylsulfonyl fluoride. The cellular extract was then centrifuged 700g for 5 minutes. The supernatant was extracted and centrifuged again at 21,000g for 15 minutes. The supernatant was extracted as the cytosolic protein fraction. The remaining cellular pellet was washed with the same Triton buffer and centrifuged at 21,000g for 15 minutes. The supernatant was discarded and the cellular pellet was washed with radioimmunoprecipitation assay buffer containing 1 mmol/L phenylmethylsulfonyl fluoride. The washed pellet solution was centrifuged at
Mitochondrial morphology changes in rMC-1 grown in HG conditions. (A) Representative confocal images of rMC-1 grown in N or mannitol (man) medium and stained with MTR show long, tubular networks of mitochondria; rMC-1 grown in HG medium for 7 days show significant mitochondrial fragmentation. Scale bars: 10 μm. (B) Form factor and AR. The graph shows average FF and AR values for mitochondria of rMC-1 grown in N, HG, or man medium for 7 days. Mitochondrial fragmentation was significantly increased in rMC-1 grown in HG medium compared to that of cells grown in N medium as indicated by decreased FF and AR values. *N versus HG; P < 0.01, n = 12. **HG versus man; P < 0.05, n = 12.

Differential Dye Staining Assay

Apoptotic cells were determined using differential dye staining, based on the cell membrane’s integrity to uptake fluorescent DNA binding dyes, ethidium bromide and acridine orange. We exposed rMC-1 grown on coverslips in N or HG medium for 7 days to a dye mixture containing 25 μg/mL ethidium bromide (Sigma-Aldrich Corp.) and 25 μg/mL acridine orange (Sigma-Aldrich Corp.) for 10 minutes, washed with PBS, fixed and mounted in a commercial reagent (SlowFade Antifade Kit; Invitrogen, Eugene, OR, USA). The cells were then visualized with a DAPI filter and at least 10 random fields were imaged using a digital camera attached to a fluorescence microscope (Diaphot; Nikon Corp.). The number of apoptotic cells per field was expressed as a percentage of the total number of cells in the field. Apoptotic cells appear orange or bright green while viable cells appear uniformly dark green.

Statistical Analysis

All data are expressed as the mean ± SD, and comparisons between the two groups were performed with Student’s t-test. Comparisons between the three groups were performed using 1-way ANOVA followed by Tukey’s post hoc test. A value of P < 0.05 was considered statistically significant.

Results

HG Exposure Induces Mitochondrial Network Fragmentation in rMC-1

We grew rMC-1 in N or HG medium for 7 days and stained with MTR before live examination by confocal microscopy to visualize the mitochondrial network. Mitochondrial network of rMC-1 grown in normal medium was interconnected and extensive throughout the cells. Individual mitochondria appeared long, tubular, and highly branched when grown in N conditions (Fig. 1A). However, the mitochondrial network of cells grown in HG conditions was significantly disturbed and scattered (Fig. 1A). Cells grown in HG medium had average FF and AR values that were significantly decreased compared with those grown in N medium (Fig. 1B; P < 0.01). Osmotic control using 30 mM mannitol for 7 days showed no change compared to those grown in normal condition (Figs. 1A, 1B).

HG Increases Membrane Potential Heterogeneity in Mitochondria of Müller Cells

To assess changes in mitochondrial membrane potential of Müller cells grown in HG medium for 7 days, rMC-1 were simultaneously stained with TMRE and MTG. Mitochondrial membrane potential heterogeneity was evaluated to investigate the distribution of mitochondrial membrane potential within individual cells. The fluorescence intensity deviation of several mitochondria within a single cell was measured, and Müller cells grown in HG showed greater variation in mitochondrial membrane potential, as seen by the greater range of TMRE and MTG overlap compared with those grown in normal medium (Fig. 2A). Mitochondrial membrane potential heterogeneity was significantly increased in Müller cells grown in HG for 7...
High Glucose and Mito Dysfunction in Müller Cells

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DISCUSSION

In this study, we examined whether HG-induced mitochondrial morphology changes promote mitochondrial dysfunction and thereby contribute to Müller cell death. Our results indicate that HG exposure induces mitochondrial fragmentation and concomitantly increases membrane potential heterogeneity, decreases oxygen consumption rate, and decreases extracellular acidification rate. Importantly, these changes are concomitant with cytochrome c release and apoptosis of rMC-1, which suggests the possibility that mitochondrial dysfunction is involved in HG-induced Müller cell death.

Our results suggest that in the presence of HG, Müller cell mitochondria undergo fragmentation with altered mitochondrial membrane potential and lowered capacity for cellular respiration, as indicated by decreased steady state and maximal oxygen consumption. While cells grown in HG also showed significant decrease in basal ECAR, it is unclear if this difference was due to glycolysis alone or in conjunction with nonglycolytic processes. Interestingly, our results also suggest that rMC-1 grown in HG have preserved respiratory and glycolytic reserves concomitant with reductions in basal OCR and ECAR. Overall, these results suggest that the respiratory capacity of retinal Müller cells is compromised in HG conditions despite the cell maintaining a degree of metabolic reserve.

Although the exact mechanisms underlying mitochondrial fragmentation in HG conditions is not completely understood, recent studies suggest that an alteration in mitochondrial fusion-fission dynamics may play an important role in compromising mitochondrial morphology. In vascular cells for instance, mitochondrial fragmentation was observed alongside an upregulation in mitochondrial fission proteins.27 Other studies have suggested that changes in mitochondrial fission or fusion may alter mitochondrial respiration, membrane potential, and cytochrome c release.28,29 More recently, studies have examined the possibility that compromised mitochondrial dysfunction, which is involved in the recycling of depolarized mitochondrial fragments,30 may occur in diabetes and promote mitochondrial dysfunction.51,52 Further studies are needed to identify specific fission and fusion genes that may be involved in HG- or diabetes-induced mitochondrial fragmentation, and to better understand mechanisms involving compromised mitochondria.

Our study shows that mitochondrial dysfunction may underlie Müller cell loss under HG conditions as demonstrated by an increasing body of studies that implicate the mitochondrion as a critical player in the pathogenesis of diabetic retinopathy.5-21,33-34 Although it is well established that mitochondria are key components of energy homeostasis and critical regulators of apoptosis in various cell types, this is the first study that shows the involvement of HG-induced mitochondrial dysfunction in retinal Müller cells. Studies have shown that increased oxidative stress contributes to mitochondrial dysfunction and ultimately retinal vascular apoptosis.53 and that mitochondrial superoxide dismutase may be protective against mitochondrial dysfunction.55,56 A study emphasized the importance of mitochondrial structural and transport proteins in the retinas of diabetic rats and those of human eyes with diabetic retinopathy.54 Additionally, studies have reported compromised mitochondrial function, such as cellular oxygen consumption under HG conditions.5-21 Furthermore, evidence of mitochondrial DNA damage and epigenetic changes such as hypermethylation of mitochondrial DNA have also been reported in HG conditions and in patients with diabetic retinopathy.57-59 Taken together, these findings suggest that mitochondrial abnormalities play a central role in the pathogenesis of diabetic retinopathy, and future therapies.
targeting mitochondrial processes may be of significant importance in treating this disease.

A limitation of the study is the use of a Müller cell line; as such, it is uncertain whether the phenomenon observed in the cell line occurs in vivo conditions. However, an advantage in using rMC-1 is that several metabolic studies in the diabetic retinopathy field have established its authenticity and a recent study demonstrated that these cells exhibit retinal Müller cell characteristics. In diabetes, it is certainly of interest to study mitochondrial morphologic changes in vivo. While it would be interesting to extend our findings with studies in an experimental animal model of diabetes, mitochondrial fragmentation can be determined in live cells in vitro using mitochondrial dyes and live-cell confocal microscope imaging, and this approach is currently not suited to assess mitochondrial fragmentation in vivo.

In diabetic retinopathy, injury to or loss of retinal Müller cells may lead to disruption in the exchange of essential metabolites between the blood and retina. This is particularly relevant in the context of diabetic retinopathy, where hyperglycemia leads to increased glucose uptake and metabolism, which can overwhelm the capacity of Müller cells to maintain normal metabolic and functional states.

**FIGURE 3.** Effects of HG on OCR and ECAR in rMC-1. (A) Decreased OCR in rMC-1 grown in HG medium. Steady-state OCR and ECAR were measured at the fifth time point. Oligomycin (O) was injected to inhibit ATP synthase, with the addition of FCCP (F) to uncouple mitochondria and obtain maximal oxygen consumption rate at the 11th time point. Finally, rotenone and antimycin A were injected (A) to confirm that the respiration changes were due mainly to mitochondrial respiration. The line graph shows the cumulative OCR data for rMC-1 grown in normal or HG for 7 days. The oxygen consumption rate was significantly decreased under HG conditions compared to normal conditions in both steady state and maximal OCR. There was no significant difference in respiratory reserve. *P < 0.03, n = 5. (B) Decreased ECAR in rMC-1 grown in HG medium. The line graph shows the cumulative ECAR data for rMC-1 grown in normal or HG for 7 days. Lines O, F, and A indicate injections of oligomycin, FCCP, and antimycin A/rotenone, respectively. Steady-state ECAR was significantly decreased under HG compared to N conditions. There was no significant difference in glycolytic reserve. *P < 0.02, n = 4.

**FIGURE 4.** High glucose exposure increases cyt c levels in retinal Müller cells. (A) Cytosolic (cyto) and mitochondrial (mito) protein fractions were isolated from rMC-1 grown in N or HG medium for 7 days and cytchrome c release in the cytosol was assessed by Western blot analysis. Purity/loading of the mitochondrial fraction was confirmed by VDAC-1 staining, and purity/loading of the cytosolic fraction was confirmed by actin staining. (B) Graph illustration showing cumulative data of rMC-1 grown in HG medium for 7 days showed increased level of cytchrome c in the cytosol. Data are normalized to the actin signal. *P < 0.05, n = 4.
High glucose promotes apoptosis in retinal Müller cells. (A) TUNEL staining (arrowhead) of rMC-1 grown in N or HG medium for 7 days, with respective DAPI stain and phase-contrast image. Scale bars: 100 µm. (B) Under HG conditions, rMC-1 grown in HG showed a significant increase in the number of TUNEL-positive cells compared to those of normal cells. *P < 0.005, n = 4. (C) Representative images of rMC-1 grown in N or HG medium subjected to differential dye staining. Scale bars: 50 µm. (D) Graph of cumulative data indicates rMC-1 grown in HG for 7 days shows significant increase in the number of apoptotic cells compared to those of N cells. *P < 0.01, n = 4.

metabolic nutrients necessary to protect retinal neurons. 41 When Müller cells become activated and undergo reactive gliosis, 42 this protective mechanism may be compromised. Additionally, in response to diabetic pathologic changes, rMC-1 produce excess VEGF 43,44 promoting retinal inflammation, neovascularization, vascular leakage, and vascular lesions. 44 Such biochemical changes in rMC-1 and retinal neurons are irreversible; therefore, injury to Müller cells in the context of diabetic retinopathy will likely result in retinal damage and ultimately disrupt retinal homeostasis. 45 Findings from this study underscore the importance of mitochondrial function in Müller cell survival and that HG-induced changes in mitochondrial bioenergetics may promote Müller cell death in diabetic retinopathy.

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