High Glucose Induces Mitochondrial Morphology and Metabolic Changes in Retinal Pericytes

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PURPOSE. Mitochondrial dysfunction is known to play a role in retinal vascular cell loss, a prominent lesion of diabetic retinopathy. High glucose (HG) has been reported to induce mitochondrial fragmentation and dysfunction in retinal endothelial cells, contributing to apoptosis. In this study, the effects of HG on mitochondrial morphology, membrane potential, and metabolic changes and whether they could contribute to HG-induced apoptosis in retinal pericytes were investigated.

METHODS. Bovine retinal pericytes (BRPs) were grown in normal or HG medium for 7 days. Both sets of cells were double stained with mitochondrial membrane potential-independent dye and tetramethylrhodamine-ethyl-ester-perchlorate (TMRE) and imaged by confocal microscopy. The images were analyzed for average mitochondria shape, by using form factor and aspect ratio values, and membrane potential changes, by using the ratio between the red and green dye. BRPs grown in normal or HG medium were analyzed for transient changes in oxygen consumption and extracellular acidification with a flux analyzer and apoptosis by TUNEL assay.

RESULTS. BRPs grown in HG media exhibited significant fragmentation of mitochondria and increased membrane potential heterogeneity compared with the BRPs grown in normal medium. Concomitantly, BRPs grown in HG showed reduced steady state and maximum oxygen consumption and reduced extracellular acidification. Number of TUNEL-positive pericytes was increased in HG condition as well.

CONCLUSIONS. In HG condition, mitochondria of retinal pericytes display significant fragmentation, metabolic dysfunction, and reduced extracellular acidification. The detrimental effects of HG on mitochondrial function and cellular metabolism could play a role in the accelerated apoptosis associated with the retinal pericytes in diabetic retinopathy. (*Invest Ophthalmol Vis Sci.* 2011;52:8657–8664) DOI:10.1167/iovs.11-7934

Hyperglycemia-induced apoptosis has been implicated as the underlying cause of vascular and neuronal cell death in the diabetic retina.^{1–3} The death of endothelial cells and pericytes in the early stages of diabetic retinopathy leads to acellular capillaries and pericytes ghosts, respectively, which

in turn accelerate the development of structural lesions characteristic of diabetic retinopathy.^{4–6} In addition, capillary degeneration as a result of vascular cell loss can lead to increased permeability and play a role in triggering the neovascularization associated with later stages of retinopathy. Thus, understanding the mechanism by which hyperglycemia or HG accelerates apoptosis of endothelial cells and pericytes in the diabetic retina is vital before a therapeutic intervention can be developed.

Pericytes are smooth-muscle-like cells with contractile properties, and they provide structural integrity to the retinal microvasculature. Importantly, loss of retinal pericytes has been hypothesized to be the initial lesion to form during diabetes,^{7,8} which can influence vessel stability, endothelial proliferation, and angiogenesis contributing to the progression of diabetic retinopathy.⁹ Several mechanisms have been proposed to explain the accelerated apoptosis of retinal pericytes under HG or diabetic condition. Increased advanced glycation end products,^{10,11} polyol pathway activation,^{8,12} upregulation of protein kinase C and TNF- α ,^{13–16} and oxidative stress¹⁷ have all been identified as mechanisms underlying the apoptosis of retinal pericytes associated with diabetic retinopathy. However, it remains unclear how HG triggers such detrimental changes in retinal pericytes, leading to apoptosis.

Oxidative stress is known to increase in diabetic retinas and trigger proapoptotic actions of mitochondria, including the release of cytochrome *c*.^{18,19} In particular, oxidative stress is known to be a key regulator of retinal vascular cell injury during HG insult, and mitochondrial dysfunction has been shown to be an agonizing factor in reactive oxygen species (ROS) overproduction in an HG or diabetic condition.¹⁹ Although mitochondrial DNA mutations,^{20,21} Bax accumulation,¹⁹ and changes in the ROS scavenging enzymes^{22,23} are documented characteristics of mitochondrial dysfunction in retinal vascular cells, it is unclear whether mitochondrial morphology changes play a role in promoting mitochondrial dysfunction in diabetic retinopathy.

In various cell types, including rat hepatocytes, myoblasts, ventricular myocytes, bovine aortic endothelial cells, and mouse smooth muscle cells, exposure to HG has been shown to induce mitochondrial fragmentation.^{24,25} Recently, we have shown that HG induces mitochondrial fragmentation and metabolic changes, leading to apoptosis in rat retinal endothelial cells.²⁶ However, it is unknown whether mitochondrial morphology is affected by HG in retinal pericytes and whether HG affects mitochondrial oxygen consumption, an index for mitochondrial metabolic activity.^{24,25,27} The purpose of this study was to establish whether apoptosis in retinal pericytes involves changes in mitochondrial shape, membrane potential, oxygen consumption, and extracellular acidification and whether these changes are attributable to the effect of HG.

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MATERIALS AND METHODS

Isolation of Bovine Retinal Pericytes

Bovine retinal pericytes (BRPs) were isolated from bovine retinas according to the protocol established by D'Amore.²⁸ Briefly, 10 to 12 adventitia-free bovine eyes were soaked in 50% betadine-2× antibiotic/antimycotic (Invitrogen-Gibco, Carlsbad, CA) PBS solution for 30 minutes. The eyes were rinsed in 2× antibiotic/antimycotic-PBS, and the retinas were removed after the eye was cut open around the sclera 3 mm away from the limbus, followed by removal of the retina from the posterior chamber by gentle scraping. Then, the retina was minced and digested in a solution of 0.2% collagenase type II (Worthington, Freehold, NJ), 0.4% BSA in unsupplemented 5 mM DMEM (Gibco) at 10 mL per six retinas for 60 minutes, with shaking at 37°C. After digestion, the retinas were passed through a 100-μm nylon mesh (Tetko, Briarcliff Manor, NY) and resuspended in 20 mL of media containing 10% FBS (Sigma-Aldrich, St. Louis, MO), 2 mM L-glutamine (Invitrogen-Gibco), and 2× antibiotic/antimycotic (Invitrogen-Gibco) in low-glucose DMEM (Invitrogen-Gibco). Then the cell suspension was centrifuged at 800g and resuspended in 10 mL of the same media to quench the collagenase. The final wash was not removed, and the cell suspension was plated with three retinas per 60-mm plastic tissue culture-treated plate (Primaria; Falcon-BD Labware, Bedford, MA) and placed in a humidified 5% CO₂ incubator at 37°C. BRPs were grown to confluence and then split at a 1:3 ratio and used in the experiments. All experiments were performed with passage 3 to 5 cells.

Cell Culture

BRPs were grown on poly-D-lysine-coated, glass slide-bottomed dishes (MatTek, Ashland, MA) in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Sigma-Aldrich), antimycotics, and antibiotics. To determine the sustained effect of HG on mitochondrial morphology and membrane potential in BRPs, cells were grown for 7 days in normal (5 mM) or HG (30 mM) medium or mannitol (30 mM) for osmotic control. Before imaging, the cells were subjected to various stains and examined by confocal microscopy.

Fluorescent Probes

To determine the sustained effect of HG on mitochondrial morphology and membrane potential heterogeneity, BRPs grown in normal or HG medium for 7 days were incubated at 37°C in a 5% CO₂ humidified chamber with 125 nM membrane potential-independent dye (MitoTracker Green [MTG]) and 8 nM TMRE, a membrane potential-dependent dye for 45 minutes, washed three times, and incubated in medium containing TMRE for 15 minutes before imaging. The latter step allows adequate equilibration of the membrane potential-sensitive TMRE dye within the mitochondria. MTG stains mitochondria green, whereas TMRE stains mitochondria red under appropriate excitation wavelength. The double-staining approach facilitates proper identification of fluorescence intensity from mitochondria at different *z*-planes. TMRE was kept in the medium while imaging. All dyes were obtained from (Invitrogen-Molecular Probes, Eugene, OR).

For time-course determination of HG's effects on mitochondrial morphology, BRPs grown in normal medium were incubated at 37°C in a 5% CO₂ humidified chamber with 8 nM TMRE in the normal medium for 10 minutes. TMRE was kept in the medium, and the cells were imaged to examine the morphology of mitochondria. To examine the effects of an HG condition on mitochondrial morphology changes, we added glucose to the medium to reach a final concentration of 30 mM. Images of random fields were then captured at the time points 0, 15, 30, 45, 60, 90, and 120 minutes.

Confocal Microscopy

Cells were imaged live by confocal microscope (LSM 510 Meta; Carl Zeiss Meditec, Oberkochen, Germany) with a 60× oil immersion objective. The cells were kept at 37°C in a 5% CO₂ humidified micro-

scope stage chamber. MTG and fluorescein isothiocyanate-conjugated secondary antibody were subjected to 488-nm argon laser excitation, and emission was recorded through a band-pass 500- to 550-nm filter. TMRE and MTG were subjected to 543-nm helium-neon laser excitation, and emission was recorded through a band-pass 650- to 710-nm filter. To observe individual mitochondria, we acquired *z*-stack images in series of six slices per cell ranging in thickness from 0.5 to 0.8 μm per slice. Fields that were imaged were selected on a random basis by moving randomly the *x*- and *y*-axis drivers on the microscope stage. The fields that had a representative number of cells were then imaged.

Mitochondrial Morphology Analysis

Quantitative analysis of mitochondrial morphology was conducted using a computer-assisted morphometric analysis application for calculation of form factor (FF) and aspect ratio (AR) values.^{29,30} Acquired images of mitochondria were analyzed using NIH-developed Image J software (Wayne Rasband; National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>) by first processing with a median filter to obtain isolated and equalized fluorescent pixels. Mitochondria were subjected to particle analysis for acquiring FF values ($4\pi \cdot \text{area}/\text{perimeter}^2$) and AR values derived from lengths of major and minor axes. An AR value of 1 indicates a perfect circle, and as mitochondria elongate and become more elliptical, AR increases. An FF value of 1 corresponds to a circular, unbranched mitochondrion, and higher FF values indicate a longer, more branched mitochondrion. For determination of the percentage of cells with fragmented mitochondria, a cell was determined to have fragmented mitochondria if it had $\geq 50\%$ of its mitochondria with $\text{FF} < 2.5$.

Mitochondrial Membrane Potential Heterogeneity Analysis

To characterize mitochondrial membrane potential heterogeneity in BRPs, a ratiometric image-analysis approach was used by dual staining with the membrane potential-dependent dye TMRE and the membrane potential-independent dye MTG. TMRE is highly permeable across the mitochondrial membrane, and so equilibration is rapid and at low concentrations, TMRE does not inhibit mitochondrial respiration. The ratio product of TMRE dye to MTG dye maintains the voltage dependency of TMRE and is independent of the exact focal plane. Thus, even though the fluorescence intensities of TMRE and MTG are variable, the ratio of fluorescence intensity of TMRE to MTG dye is essentially independent of the focal plane.³¹

Images were analyzed for membrane potential of individual mitochondrion using deviation of fluorescence intensities for the ratio of red (TMRE) to green (MTG) dye for several mitochondria within each cell. The relative membrane potential of a single mitochondrion within a cell was calculated by applying modified versions of the Nernst equation: $E_{\text{TMRE}} = (-61/z) \log(\text{TMRE}_{\text{in}}/\text{TMRE}_{\text{out}})$, where *z* is charge of TMRE (+1). Within each cell, the SD of all mitochondrial membrane potentials was calculated to derive the overall membrane potential heterogeneity.

Cellular Oxygen Consumption and Extracellular Acidification

The oxygen consumption and extracellular acidification rates of BRPs grown in normal or HG medium for 7 days was measured by a bioenergetic assay (XF24; Seahorse Bioscience, Billerica, MA), as previously described.³² Briefly, BRPs were plated and grown on 24-well microplate for 7 days in normal or HG medium to assess cellular oxygen consumption and extracellular acidification rates. Assays were initiated by removing growth medium, replacing with low-buffered RPMI 1640 medium containing 1 mM phosphate (Molecular Devices, Sunnyvale, CA) and incubating at 37°C for 60 minutes to allow temperature and pH to reach equilibrium. The microplate was then assayed (XF24 Extracellular Flux Analyzer; Seahorse Bioscience), to measure extracellular flux changes of oxygen and pH in the medium immediately surrounding the adherent cells. After steady state oxygen consumption and extracellular acidification rates

were obtained, oligomycin (5 μ M), which inhibits ATP synthase, and the proton ionophore FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazide; 1 μ M), which uncouples mitochondria, were injected sequentially through reagent delivery chambers for each cell well in the microplate, to obtain the maximum oxygen consumption rates. Finally, a mixture containing 5 μ M rotenone (an inhibitor of mitochondrial complex I) and 5 μ M antimycin A (an electron transport blocker) was injected to confirm that respiration changes were due mainly to mitochondrial respiration.

The values of oxygen consumption and extracellular acidification reflect the metabolic activities of the cells and the number of cells, so oxygen consumption and extracellular acidification rates were normalized to the total amount of cells in each well.

Western Blot Analysis

BRPs grown in normal and HG conditions were washed with PBS and lysed with 0.1% Triton X-100 buffer containing 10 mM Tris (pH 7.5), 1

mM EDTA, and 1 mM PMSF. An equal volume of 2 $\times$ sample buffer was added to the protein samples followed by denaturation at 95°C for 5 minutes. Then, the protein samples were electrophoresed at 120 V for 50 minutes. Kaleidoscope molecular weight standards were run in separate lanes in each gel. After completion of electrophoresis, the protein samples were transferred to nitrocellulose membranes by using a semidry apparatus with a Towbin buffer system, according to a procedure published by Towbin et al.³³ The membranes were blocked with 5% nonfat dry milk for 1 hour and then exposed to rabbit anti-VDAC1 (an abundant, outer mitochondrial membrane protein that acts as a nonselective anion channel for the mitochondrial outer membrane, Abcam, Cambridge, MA) or rabbit anti- β -tubulin (Cell Signaling, Danvers, MA) in 0.2% nonfat milk overnight. After overnight incubation, the blots were washed with Tris-buffered saline containing 0.1% Tween-20 and then incubated with anti-rabbit IgG secondary antibody (Sigma-Aldrich, St. Louis, MO) for 1 hour. The membrane was again washed as above and then exposed to a chemiluminescent

FIGURE 1. Seven days of HG exposure induced mitochondrial fragmentation in retinal pericytes. (A) Confocal images of BRPs grown in normal (N) medium showing interconnected network of long, tubular mitochondria throughout the cytoplasm. (B) BRPs grown in HG medium for 7 days showed significant disruption of the mitochondrial network and mitochondrial fragmentation. Scale bar, 5 μ m. (C) BRPs grown in 30 mM mannitol for 7 days showed normal mitochondrial morphology. (a'–c') FF versus AR values for individual mitochondria within BRPs grown in normal or HG medium for 7 days. BRPs grown in normal medium or 30 mM mannitol had greater FF and AR values, corresponding to a long, highly branched shape. In the HG condition, mitochondria of BRPs clustered toward lower FF and AR values, indicating fragmentation and punctate shape. (D) Of the total pericytes observed, a significant percentage of BRPs grown in HG for 7 days had fragmented mitochondria, compared with those grown in normal medium. (E) Average FF and AR values for mitochondria of BRPs grown in normal or HG medium for 7 days. Mitochondria were significantly fragmented in BRPs grown in HG medium, as shown by the decreased FF and AR values. * $P < 0.01$.

protein detection system (Immun-Star Chemiluminescent; Bio-Rad, Temecula, CA) to detect the protein signals on an x-ray film. Densitometry was conducted and analyzed (NIH Image, Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>).

Terminal dUTP Nick-End Labeling (TUNEL)

To determine apoptosis, a TUNEL assay was performed on BRPs grown in normal or HG medium (ApopTag In Situ Apoptosis Detection kit; Chemicon, Temecula, CA), according to the manufacturer's instructions. Briefly, cells grown on coverslips were fixed with 4% PFA and permeated with a precooled mixture of 2:1 ratio of ethanol to acetic acid. After two washes in PBS, the cells were incubated with equilibration buffer and then incubated with TdT enzyme in a moist chamber at 37°C for 1 hour. The cells were then washed with PBS and incubated with antidigoxigenin peroxidase. Finally, the cells were washed in PBS and mounted (SlowFade; Molecular Probes). Images from 10 random fields representing each coverslip were captured with a digital microscope and recorded for analysis (DS-Fi1; Nikon, Tokyo, Japan).

Statistics

All data are expressed as the mean $\pm$ SD. Comparisons between groups were performed with Student's *t*-test. *P* < 0.05 was considered statistically significant.

RESULTS

Fragmentation of the Mitochondrial Network in Retinal Pericytes Grown in HG for 7 Days

BRPs were cultured in normal or HG medium for 7 days, stained with TMRE, and examined live by confocal microscopy to examine mitochondrial morphology. Pericytes grown in normal glucose had an extensive network of mitochondria throughout the cell, and the shape of individual mitochondria is long, tubular, and highly branched (Fig. 1A). When grown in HG condition, the mitochondrial network of the pericyte appeared significantly disrupted (Fig. 1B). The observed disruption of the mitochondrial network was not due to an osmotic effect, as 30 mM mannitol treatment for 7 days did not disrupt the mitochondrial morphology (Fig. 1C). Accordingly, the FF and AR values, which measure length and branching of the mitochondria, were clustered at lower values for mitochondria of pericytes grown in HG (Fig. 1b' vs. 1a' and 1c'), and the average FF and AR values were significantly decreased compared with normal (Fig. 1E; FF = 3.00, compared with 4.71 in normal medium, *P* < 0.001; AR = 2.54, compared with 2.92 in normal, *P* = 0.006). In addition, a higher percentage of the pericytes showed fragmented mitochondria (Fig. 1D; 41.8% $\pm$ 9.5% of mitochondria vs. 16.8% $\pm$ 4.4% in normal; *P* = 0.004).

Acute HG Exposure Causes Transient Mitochondrial Fragmentation from Which Retinal Pericytes Recover

When BRPs were exposed acutely to the HG condition, mitochondrial fragmentation was observed within 30 minutes of HG exposure (Fig. 2; FF = 3.16 compared with 4.88 in normal, *P* < 0.001; AR = 2.47 compared with 2.92 in normal, *P* = 0.015). Mitochondrial fragmentation persisted in HG condition through the 2-hour time point, but at six hours of HG exposure BRPs significantly recovered from the mitochondrial fragmentation, as mitochondrial morphology returned to near-normal levels (Fig. 2; FF = 4.35, *P* < 0.001; AR = 2.95, *P* = 0.03). Normal mitochondrial morphology continued until 48 hours of HG exposure, when mitochondrial fragmentation was observed again (Fig. 2; FF = 3.65, *P* < 0.001; AR = 2.70, *P* =

FIGURE 2. Acute HG exposure induces transient changes in mitochondrial morphology. (A) Confocal images showing mitochondria of BRPs exposed to an HG condition for the indicated time points. Scale bar, 5 μ m. (B) Average FF (■) and AR (□) values for mitochondria of BRPs grown in normal medium and then exposed to HG medium for 0, 30, or 60 minutes or 2, 6, 24, or 48 hours. A significant decrease in FF and AR values was observed at 30 m of HG, indicating mitochondrial fragmentation, and continued through 2 hours. Recovery from HG-induced mitochondrial fragmentation was seen at 6 and 24 hours, but mitochondria became fragmented again at 48 hours. **P* < 0.01 compared to 0 minutes of HG; ***P* < 0.01 compared to 30 minutes of HG.

0.04), and the fragmented mitochondria persisted through 7 days of HG exposure. Thus, HG induces a bi-phasic change in mitochondrial morphology: a transient mitochondrial fragmentation, from which pericytes can recover, followed by permanent mitochondrial fragmentation.

Altered Membrane Potential in Retinal Pericytes Grown in HG

To assess changes in mitochondrial membrane potential of BRPs grown in HG medium for 7 days, pericytes were double stained with TMRE and MTG. Since TMRE is a membrane potential-sensitive dye, and MTG is independent of membrane potential, the ratio of the two dyes gives a value for membrane potential: More red color indicates increased uptake of TMRE and thus a higher membrane potential. BRPs grown in HG showed decreased membrane potential compared with pericytes grown in normal medium (Fig. 3B vs. 3A, 3C; 84% $\pm$ 11% of control; *P* = 0.04).

To assess whether HG-induced mitochondrial fragmentation affects the distribution of mitochondrial membrane potential within a cell, mitochondrial membrane potential heterogeneity was quantified by measuring the fluorescence intensity deviation of several mitochondria within a single cell. When grown in HG, pericytes showed greater variation in mitochondrial membrane potential within a cell (Fig. 3B), as seen by the greater range of green (depolarized) to red (hyperpolarized)

FIGURE 3. Mitochondrial membrane potential changes of retinal pericytes grown in HG. (A, B) BRPs grown for 7 days in normal (N) or HG medium were double stained with mitochondrial membrane potential-sensitive TMRE (red) dye and membrane potential-independent MTG (green) dye to observe changes in mitochondrial membrane potential. Scale bar, 10 μ m. (a', b') Pseudocolored images of (A) and (B) that enhance the variation in mitochondrial membrane potential within the cell. (C) The average mitochondrial membrane potential of BRPs grown in normal (N) or HG medium for 7 days. In an HG condition, BRP mitochondria have reduced average membrane potential. (D) The graph shows the SD of the fluorescence intensity membrane potentials for several mitochondria within BRPs grown in normal (N) or HG medium for 7 days. BRPs grown in HG had a greater SD of the membrane potentials indicating a significant increase in the heterogeneity of mitochondrial membrane potential. * $P < 0.05$.

color compared with those grown in normal medium (Fig. 3A). To demonstrate this distinction, ratiometric images were produced by coding the fluorescence intensity. Figures 3a' and 3b' are pseudocolored images of Figure 3A and 3B and allow improved visualization of mitochondrial membrane potential heterogeneity. Mitochondrial membrane potential heterogeneity was significantly increased in BRPs grown in HG for 7 days compared with those grown in normal medium (Fig. 3C; $202\% \pm 39\%$ of control, $P = 0.003$). Therefore, HG conditions not only disrupt mitochondrial morphology by fragmenting the mitochondrial network, but mitochondrial membrane potentials within each pericyte are more heterogeneous than normal.

HG Decreases Steady State Maximum Oxygen Consumption and Extracellular Acidification in Retinal Pericytes

BRPs grown in normal or HG conditions were measured simultaneously with a bioenergetic assay (XF24; Seahorse Bioscience) to determine rates of cellular oxygen consump-

tion and extracellular acidification. Steady state oxygen consumption and extracellular acidification were measured at the fourth time point (Fig. 4A). Oligomycin (Fig. 4A; injection vertical line A) was injected to inhibit ATP synthase, followed by the addition of FCCP (Fig. 4A: injection vertical line B) to uncouple mitochondria and obtain values for maximum oxygen consumption. Finally, rotenone and myxothiazol were injected (Fig. 4A; injection vertical line C) to confirm that the respiration changes could be attributed to mitochondrial respiration. BRPs grown in HG showed a significant decrease in steady state (Fig. 4B; steady state: 36.2 vs. 50.0 pmol O_2 /min/ 10^6 cells in normal; $P = 0.012$) and maximum oxygen consumption (maximum 88.1 vs. 132 pmol O_2 /min/ 10^6 cells in normal; $P = 0.03$, $n = 6$), compared with pericytes grown in normal medium.

Extracellular acidification rates were examined simultaneously for BRPs grown in HG. Changes in the extracellular acidification rate may indicate changes in the rate of glycolysis in the pericytes. Under HG exposure, BRPs showed significantly decreased extracellular acidification compared with pericytes grown in normal medium (Fig. 4C; $60.5\% \pm 24\%$ of normal; $P = 0.018$, $n = 6$). The

FIGURE 4. HG affects mitochondrial oxygen consumption and extracellular acidification in retinal pericytes. (A) The line graph shows the experimental evaluation of steady state and maximum oxygen consumption (OCR) for BRPs grown in normal or HG for 7 days (white circles/dashed line, normal; black squares/dark line, HG). Dotted lines A, B, and C indicate injections of oligomycin, FCCP, and rotenone/antimycin A, respectively. Steady state oxygen consumption was measured at the fourth time point before oligomycin injection, whereas maximum oxygen consumption was measured at the 10th time point after FCCP injection. (B) HG exposure of 7 days resulted in reduced steady state and maximum oxygen consumption rates of BRPs. (C) The extracellular acidification rate (ECAR) was measured at the fourth time point and was significantly decreased after 7 days of HG exposure compared with normal. (D) VDAC1 protein levels of BRPs grown in normal (N) or HG medium for 7 days. No change in VDAC1 expression (corrected to β -tubulin expression) was observed after 7 days of HG, indicating that total mitochondrial content is not affected. * $P < 0.05$.

results may indicate that BRPs grown in HG cannot compensate for HG-induced decreases in metabolic capacity, as displayed by decreased oxygen consumption.

Rates of cellular oxygen consumption may be affected by the number of mitochondria; thus, to assess the mitochondrial content of BRPs grown in normal or HG medium for 7 days, whole-cell extract was assessed for VDAC1 expression by Western blot analysis. VDAC1 expression showed no change after HG exposure (Fig. 4D; $n = 6$), suggesting no change in mitochondrial content. Thus, the data suggest that BRPs grown in HG showed significant mitochondrial fragmentation and subsequent decrease in mitochondrial metabolic capacity, as indicated by decreased oxygen consumption. Also, the VDAC1 data may support the idea that HG causes increased fragmentation of the mitochondria, resulting in more mitochondrial units per cell, but the overall mitochondrial content remains unchanged. However, more direct assessment of mitochondrial volume changes that occur in the HG condition are needed to substantiate this claim.

Number of TUNEL-Positive Cells Increases with HG-Induced Mitochondrial Morphology and Metabolic Changes

To determine whether changes in mitochondrial shape and metabolism were concomitant with increased apoptosis, TUNEL-staining was performed on BRPs grown in normal or HG. The number of TUNEL-positive cells was significantly increased in BRPs grown in HG compared with those grown in

normal medium (Figs. 5A, 5B; 5.6 ± 2.6 TUNEL-positive cells/1000 cells compared with 13.6 ± 3.5 TUNEL-positive cells/1000 cells in normal; $P = 0.007$).

DISCUSSION

We sought to investigate whether HG condition has an effect on mitochondrial morphology and function in retinal pericytes and whether such changes are associated with increased apoptosis. Pericyte loss is a well-documented pathology of the retinal capillaries during the early stages of nonproliferative diabetic retinopathy.⁶ Understanding the mechanisms by which hyperglycemia accelerates apoptosis of retinal pericytes may help identify novel targets for therapeutic intervention. The results from this present study indicate that exposure to HG caused a fragmentation in retinal pericytes, and prolonged HG caused mitochondrial membrane potential and oxygen consumption to decrease, along with decreased extracellular acidification. Importantly, the mitochondrial dysfunction seen with prolonged HG exposure appeared to underlie increased apoptosis of the retinal pericytes.

Our results demonstrate for the first time that HG condition can cause dramatic changes to mitochondrial morphology in retinal pericytes. This mitochondrial fragmentation was similar to that observed in retinal endothelial cells grown in an HG condition.³⁴ The retinal pericytes also displayed a bi-phasic mitochondrial fragmentation, a rapid fragmentation in response to HG from which the pericytes recovered and a per-

FIGURE 5. HG increased the number of TUNEL-positive retinal pericytes. **(A)** TUNEL staining of BRPs grown in normal (N) or HG medium for 7 days, with respective DAPI stain and phase-contrast image. The number of TUNEL-positive cells (*arrow*) was significantly increased in BRPs grown in HG compared with those grown in normal medium. Scale bar, 25 μ m. **(B)** In an HG condition of 7 days, BRPs grown in HG showed a significant increase in the number of TUNEL-positive cells compared to those of normal cells. * $P < 0.05$.

sistent fragmentation that occurred after 48 hours of HG. Bi-phasic mitochondrial fragmentation has been reported in other studies, and it has been shown that mitochondrial fission in response to acute HG exposure influences ROS overproduction.^{24,25} Although upregulation of the ROS level is closely associated with the first round of mitochondrial fragmentation and the recovery of morphology reduces ROS levels, the exact implication of these cellular events is currently unknown. We speculate that the morphology recovery is an attempt by the cells to recover against oxidative stress through regaining mitochondrial structural integrity; however, the cells also ultimately succumb to the HG insult and permanent mitochondrial fragmentation. Permanent mitochondrial fragmentation in retinal pericytes exposed to HG condition may play a significant role in promoting mitochondrial dysfunction and subsequent apoptosis, leading to pericytes loss in diabetic retinopathy.

Recent research has demonstrated how maintenance of mitochondrial morphology is critical for regulating mitochondrial metabolism, ROS production, and induction of apoptosis.³⁵ Our previous report on retinal endothelial cells demonstrated how fragmentation of the mitochondrial network could compromise the metabolic capacity of the mitochondria in these cells when grown in an HG condition.³⁴ Similarly, our data also indicate that HG-induced mitochondrial fragmentation may play a role in decreasing mitochondrial oxygen consumption. Mitochondrial membrane potential was similarly decreased and displayed significant heterogeneity within individual cells. The observed mitochondrial membrane potential heterogeneity may represent a functional manifestation of the more disconnected mitochondrial network, which ultimately may influence apoptosis.³⁶ Consequently, mitochondrial morphology changes in both retinal endothelial cells and pericytes may exacerbate mitochondrial functional changes and the progression to apoptosis in an HG or diabetic condition.

The cause-effect relationship of mitochondrial fragmentation has recently been reported in endothelial cells derived from diabetic patients.³⁷ Mitochondrial fragmentation was studied in the context of hyperglycemia in vascular cells, and the results showed increased mitochondrial fission as a contributing mechanism for endothelial dysfunction in a diabetic condition. In particular, *Fis1*, a gene responsible for regulating mitochondrial fission, was overexpressed in the diabetic endothelial cells and was concomitant with mitochondrial fragmentation. Furthermore, when siRNA was used to prevent HG-induced *Fis1* overexpression in a tissue culture model of diabetes, the mitochondrial fragmentation was observed to be significantly reduced, and ROS upregulation, a hallmark of

diabetes, was also inhibited. In this study, we are reporting that HG induces mitochondrial fragmentation in retinal pericytes and that the morphology change is associated with mitochondrial dysfunction and subsequent apoptosis of the pericytes.

Since mitochondrial oxygen consumption is compromised in retinal pericytes exposed to HG, we assessed whether extracellular acidification was altered to analyze any changes in glycolytic levels of the cells. In retinal endothelial cells, we reported an increase in extracellular acidification levels, possibly to compensate for HG-induced decreased mitochondrial oxygen consumption. Interestingly, the retinal pericytes displayed a significant decrease in extracellular acidification. This inability to compensate for HG-induced decrease in mitochondrial oxygen consumption may indicate an increased susceptibility of the retinal pericytes to the HG insult. In addition, the differences seen between the pericytes and endothelial cells may be explained by differential transport of glucose between the two cell types. Previous reports have shown that HG downregulates Glut1 activity in pericytes, but not in endothelial cells.³⁸ Thus, retinal pericytes and endothelial cells differentially alter extracellular acidification levels and possibly glycolytic levels in response to HG. Further research is needed to identify the mechanisms and consequences for the observed disparity.

The findings of this study indicate a novel step in the pathway for accelerated apoptosis in retinal pericytes, including a direct effect of HG on mitochondrial morphology. Permanent mitochondrial fragmentation, decreased mitochondrial metabolic capacity and extracellular acidification are observed in the pericytes grown in HG, along with increased apoptosis. The results provide further understanding of HG-induced mitochondrial dysfunction and how it may contribute to apoptosis of retinal pericytes. Preventing mitochondrial morphology changes in response to hyperglycemic environment may represent a novel therapeutic target for preventing pericyte loss in the early stages of diabetic retinopathy.

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