Mitochondrial DNA Oxidative Damage Triggering Mitochondrial Dysfunction and Apoptosis in High Glucose–Induced HRECs

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PURPOSE. Oxidative stress is linked to early apoptosis in diabetic retinopathy, and reactive oxygen species (ROS) play a critical role in that stress. Endogenous ROS generated from attacks of mitochondria on nearby mitochondrial DNA (mtDNA) could lead to constant ROS overproduction. The authors explored the role of mtDNA oxidative damage in high glucose-induced dysfunction in human retinal vascular endothelial cells (HRECs).

METHODS. mtDNA oxidative damage was examined by Southern blot analysis in HRECs treated with high glucose. The effect of mtDNA damage on the mRNA expression of respiratory chain subunits, change of mitochondrial membrane potential (ΔΨm), overproduction of ROS, and apoptosis were assessed in high glucose–treated HRECs by RT-PCR, flow cytometry, and confocal microscopy.

RESULTS. mtDNA oxidative damage increased rapidly after short-term (3-hour) exposure to high glucose (30 mM) and was severe after 48-hour exposure. Accordingly, the mRNA level of respiratory chain subunits NADPH-1 and CO1 encoded by mtDNA was significantly downregulated after exposure to high glucose (P < 0.05) compared with levels encoded by nuclear DNA. Treatment with 12-, 24-, and 48-hour high glucose resulted in decreased membrane potential and overproduction of ROS in HRECs (P < 0.05); early apoptosis was observed with 24-hour (9.63% ± 1.26%) and 48-hour (12.30% ± 1.43%) treatment.

CONCLUSIONS. mtDNA oxidative damage seems to be the “trigger” for cell dysfunction in high glucose–treated HRECs by setting in motion the vicious circle of mtDNA damage leading to ROS overproduction and further mtDNA damage, which may explain in part early vascular dysfunction in diabetic retinopathy. (Invest Ophthalmol Vis Sci. 2008;49:4203–4209) DOI:10.1167/iovs.07-1364

Diabetic retinopathy, one of the most important microvascular complications in type 1 and type 2 diabetes, has been considered a major cause of visual impairment worldwide.1,2 Many biochemical mechanisms have been proposed to explain the structural and functional abnormalities associated with overexpression of the vascular tissues to hyperglycemia.3–5 Apoptosis of retinal capillary cells is an early event in the pathogenesis of diabetic retinopathy, and oxidative stress has been linked to accelerated apoptosis of retinal capillary cells.6–8 Recently, the role of oxidative stress in the development of diabetes has been emphasized, with reactive oxygen species (ROS) considered a key factor.9,10

The mitochondrial respiratory chain is the major endogenous source of intracellular ROS and is considered a causal link between elevated glucose and the major biochemical pathways. These pathways, including elevated polyol pathway activity, non-enzymatic glycation, and protein kinase C level, were postulated to be involved in the development of vascular complications.11–12 Earlier, vitamin E, a classic antioxidant, was found to decrease oxidative production without protecting against endothelial cell dysfunction in diabetic rats and cardiovascular patients.13–14 Anti-oxidant therapy with vitamin E or other antioxidants was suggested to be limited in scavenging already-formed oxidants and may be considered to treat the symptoms more than the cause in vascular oxidative stress. Therefore, superoxide dismutase or catalase mimetics, interrupting the overproduction of superoxide by the mitochondrial electron-transport chain, demonstrated more beneficial effects.15,16

How ROS is overproduced in hyperglycemia is unclear, and antioxidative therapy, scavenging formed oxidants and interrupting the overproduction of ROS, is probably passive. Among the biomacromolecules attacked by ROS, mitochondrial DNA (mtDNA) is nearest to the respiratory chain, the site of ROS production. Furthermore, mtDNA lacks a strong repair system compared with nuclear DNA.17,18 mtDNA, encoding most of the respiratory chain proteins, is largely attacked by ROS, which results in constant overproduction of ROS. In this process, oxidative damage of mtDNA seems to be a key point. From this scenario, we suggested that oxidative damage in mtDNA could explain ROS overproduction in hyperglycemia and ROS-mediated cellular damage “memory” after glucose normalization.19

Few studies have focused on the role of mtDNA oxidative damage of human retinal vascular endothelial cells (HRECs), the first to receive the hyperglycemia signal in the retina, in the development of diabetic retinopathy. We established HRECs induced by high glucose and determined by Southern blot analysis the lesions by 8-oxo-guanine (8-oxo-G), the sensitive marker of DNA oxidative damage, in mtDNA of HRECs. mtDNA oxidative damage found in high glucose–treated HRECs could trigger mitochondrial dysfunction and apoptosis. This result furthered the understanding of the mechanism of vascular dysfunction in diabetic retinopathy and may offer a new target for therapy of diabetic vascular complications.

METHODS

HREC Culture and Treatment

Cultured HRECs from eyes of 10 donors were obtained from the Zhongshan Ophthalmic Center Eye Bank. The donors, whose mean age was 32.3 years, had been otherwise healthy victims of accidents. The procedures for harvesting retinas were previously described.20 Briefly, to avoid significant pigment epithelium contamination, the retinas were harvested carefully, placed in Hanks balanced salt solution, and
washed. After they were minced gently, the retinal pieces were digested in 2% trypsin for 20 minutes at room temperature, collected by centrifugation at 1200 rpm, and resuspended in 0.1% collagenase for 20 minutes at 37°C. The homogenate underwent centrifugation (1000 rpm, 10 minutes), and the pellet was resuspended in human endothelial-serum free medium (HE-SFM; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum, 5 ng/mL recombinant human β9252-endothelial cell growth factor (β9252-ECGF; R&D Systems, Minneapolis, MN), and 1% insulin-transferrin-selenium. Cells were cultured in fibronectin-coated flasks and incubated at 37°C in a humidified atmosphere containing 5% CO2. Cultured cells were characterized for endothelial homogeneity by testing for immunoreactivity to factor VIII antigen on light microscopy. To avoid age-dependent cellular modification, only cells at passages 2 and 3 were used.

HRECs were seeded in a Petri dish and then in a logarithmic phase were exposed to HE-SFM with 30 mM glucose for 1, 3, 6, 12, 24, and 48 hours; control cells were cultured in HE-SFM with 5 mM glucose. (We chose the 48-hour untreated HRECs as control because there was no significant change in untreated age-matched cells in our preliminary experiment.)

**Southern Blot Analysis of mtDNA Oxidative Damage**

At the indicated times, the cells of two 60-mm dishes were digested and harvested by centrifugation; cell pellets were kept in −80°C for analysis. DNA was extracted (DNeasy Blood & Tissue Kit; Qiagen, Hilden, Germany), and 10 μg purified DNA was digested with 1 μL BamHI (NEB, Hitchin, UK) overnight at 37°C. After restriction, DNA was purified by use of phenol-chloroform and dissolved in distilled water. Precise DNA concentrations were measured by use of a biophotometer. Restricted DNA (1.5 μg) was digested with 0.75 μL formamidopyrimidine DNA glycosylase (Fpg) protein (NEB). Reaction was stopped by heat at 60°C for 10 minutes. The mixture, including 1 μg DNA, was separated in a 0.8% denaturing alkaline agarose gel and transferred to a nylon membrane. The content of 8-oxo-G lesions was determined by use of a labeling and detection kit (DIG High Prime DNA Labeling and Detection Starter kit; Roche, Mannheim, Germany). The membrane was hybridized with a DIG-labeled probe overnight at 60°C. The probe for mtDNA was generated by PCR, with the human total DNA sequence used as a template. Primer sequences for the mitochondrial probe were forward, 5′TAGGGTTAGCAGCTGAAAGTT, and reverse, 5′TTCATAGTAGAAAGGCGATG. The 592-bp PCR product was hybridized with a restriction fragment of human mtDNA digested with BamHI. After hybridization, the membrane was washed and developed with DIG. Resultant band images were scanned by use of a gel imaging system (Gel-Pro Analyzer; Media Cybernetics, Bethesda, MD). Results of four experiments were pooled to calculate changes in equilibrium lesion density with the Poisson equation (s = ln B1H/B1C, where s is the number of enzyme incisions, B1H is the band intensity in high glucose-treated HRECs, and B1C is the band intensity in controls).

**FIGURE 1.** Representative quantitative Southern blot analysis of mtDNA oxidative damage in HRECs. (a) Hybridization intensity of mtDNA from HRECs treated with normal (5 mM; control, C) and high (30 mM) levels of glucose for the indicated times. (b) Calculated changes in equilibrium lesion density by 8-oxo-G. Results are mean ± SD of four experiments.

**FIGURE 2.** (a) Lanes 9 to 15: RT-PCR analysis of mRNA of NADPH-1, CO1, and ATPsynβ in controls and high glucose–treated HRECs at various times. Lanes 1 to 7: β-actin. Lane 8: 100-bp ladder. (b) Down-regulation of NADPH-1 and CO1 began at 6 and 12 hours, respectively. Normalization was β-actin (Actb) (*P < 0.05). Both subunits showed significant decreases with 48-hour exposure to high glucose (30 mM). Results are representative of experiments repeated four times.
Semiquantitative PCR

Total RNA was isolated by use of an RNA purification system (PureLink Microto-Midi Total RNA Purification System; Invitrogen, Carlsbad, CA). RNA (1 μg) was reverse transcribed by first-strand synthesis (SuperScript III First-Strand Synthesis System for RT-PCR; Invitrogen). For PCR amplification, 1 μL cDNA was denatured for 5 minutes at 94°C, then for 26 cycles for CO1 and NADPH-1 and 28 cycles for ATPsynβ. Each cycle included 1-minute denaturation at 94°C, 40-second primer annealing at 50°C for CO1, 60°C for NADPH-1, and 58.2°C for ATPsynβ, then 1-minute polymerization at 72°C. Primer sequences were as follows: NADPH-1 forward, 5′-GGCCCCAACGTTGAGGC; NADPH-1 reverse, 5′-GTCTACAGCTCTGATGACT; CO1 forward, 5′-TCCTCATAAGCACTCGTACT; CO1 reverse: 5′-GTGGTAAAGGCTCA-GAAA; ATPsynβ forward, 5′-CTCTTCCGCACGAAGCAGG; ATPsynβ reverse, 5′-TCTTGTTGTGGTATGGGACC; β-actin (Actb) forward, 5′-CTTGAAGCTGTTTG; and β-actin reverse, 5′-CCTGTAGAGTTCGTTG; and β-actin reverse, 5′-CCTGTAGAGTTCGTTG. RT-PCR products were 745, 233, 335, and 314 bp, respectively.

Flow Cytometry for Measurement of Δψm

To measure the Δψm of HRECs challenged by high glucose, we used a fluorescent probe (5′,6′-dichloro-1′,3′,3′-tetraethylbenzimidazole carboxyanide iodide; JC-1; Molecular Probes, Eugene, OR) as described. Briefly, HRECs (5 × 10^5) were collected by trypsinization, then 1-minute polymerization at 72°C. Primer sequences were as follows: NADPH-1 forward, 5′-GGCCCCAACGTTGAGGC; NADPH-1 reverse, 5′-GTCTACAGCTCTGATGACT; CO1 forward, 5′-TCCTCATAAGCACTCGTACT; CO1 reverse: 5′-GTGGTAAAGGCTCA-GAAA; ATPsynβ forward, 5′-CTCTTCCGCACGAAGCAGG; ATPsynβ reverse, 5′-TCTTGTTGTGGTATGGGACC; β-actin (Actb) forward, 5′-CTTGAAGCTGTTTG; and β-actin reverse, 5′-CCTGTAGAGTTCGTTG. RT-PCR products were 745, 233, 335, and 314 bp, respectively.

ROS Assay

Cultured cells underwent double-labeling with 20,70-dichlorodihydrofluorescein diacetate (H2DCFDA; Molecular Probes) to detect ROS production and mitochondrion-specific dye (MitoFluor Red 589; MFL, Molecular Probes) to visualize mitochondria. MFL accumulates in mitochondria regardless of mitochondrial membrane potential and has a long emission wavelength (622 nm) that is well resolved from the 525-nm emission wavelength of green fluorescein analogues such as H2DCFDA. HRECs were rinsed with PBS at different times. After H2DCFDA and MFL were added to nonphenol red culture media with final concentrations of 2 mM and 500 nM, respectively, cells were incubated at 37°C for 45 minutes, then washed twice with fresh prewarmed medium and viewed under a laser scanning confocal microscope (LSM 510; Zeiss, Oberkochen, Germany). The green fluorescence of H2DCFDA was excited at 488 nm, and that of MFL was excited at 588 nm. Mean fluorescence intensity per square millimeter cell area was calculated by use of the Zeiss software.

Flow Cytometry for Measurement of Apoptosis

Apoptosis was assessed by use of an annexin V/propidium iodide (PI) kit according to the manufacturer’s instructions (Bender Med Systems, Vienna, Austria). HRECs (5 × 10^5) were washed twice with PBS and suspended in 100 μL 1× binding buffer. Annexin V (5 μL) and 10 μL PI were added to the cell suspension, vortexed, and incubated for 15 minutes in the dark. Finally, 400 μL of 1× binding buffer was added, and samples were evaluated by flow cytometry.

RESULTS

Increased mtDNA Oxidative Damage in HRECs Treated with High Glucose

Total DNA from high glucose–treated HRECs and controls was treated with saturating concentrations of Fpg to determine the

![Figure 3](https://example.com/figure3.png)

**Figure 3.** HRECs were treated with normal (5 mM; control) and high (30 mM) levels of glucose at the indicated times for flow cytometry. (a) Cells displaying red fluorescence in UL indicate high Δψm, cells displaying both red and green fluorescence in UR indicate low Δψm, and cells displaying green fluorescence in LR indicate low Δψm than that in UR. Cells treated with high glucose for the indicated times showed migration to the right and lower quadrants. (b) The ratio of red/green fluorescence represented Δψm in HRECs with high-glucose treatment for the indicated times. Values represent mean ± SD of four independent experiments. *P < 0.05.
level of endogenous mtDNA lesion damage by 8-oxo-G, a sensitive marker of DNA oxidative damage (Fig. 1a). Treatment with high glucose (30 mM) beginning at 3 hours showed increased mtDNA damage in HRECs treated with high glucose (Fig. 1b).

**Downregulation of Respiratory Chain Subunits NADPH-1 and CO1 but Not ATPsynβ with Hyperglycemia**

To understand the mechanism of high glucose–induced cell dysfunction, we examined high glucose–induced alteration in the mRNA level of several respiratory chain subunits. The mRNA level of NADPH-1 and CO1, encoded by mtDNA, was downregulated in a time-dependent manner, beginning at 6 hours (1.04 ± 0.09) and 12 hours (0.54 ± 0.07; both P < 0.05) of treatment; at 48 hours, both subunits showed significantly decreased expression of high-glucose exposure (Fig. 2a). However, ATPsynβ, encoded by nuclear DNA, showed no significant change in expression (Fig. 2b).

**Disturbance of Δψm in HRECs Treated with High Glucose**

To determine the involvement of the mitochondrial-mediated pathway in high glucose–induced cell dysfunction, we measured Δψm by flow cytometry. High-glucose treatment of HRECs resulted in rapid deterioration in membrane potential in a time-dependent manner (Fig. 3). A significant decrease in the ratio of red to green fluorescence began at 12 hours (18.65 ± 3.82) and continued at 24 hours (8.86 ± 3.09) and 48 hours (5.44 ± 2.30; all P < 0.05) after high-glucose treatment.

**Overproduction of ROS in High Glucose–Treated HRECs**

Confocal microscopy was used to localize the high glucose–induced increase of ROS production in mitochondria of HRECs. MFL and H2DCFDA fluorescence to visualize mitochondria location and ROS production, respectively, showed high colocalization with longer exposure to high-glucose treatment, indicating that mitochondria are the major source of ROS production on exposure (Fig. 4). H2DCFDA fluorescence intensity per square millimeter cell area increased over time beginning at 12 hours (9.90 ± 1.68; P < 0.05).

**Apoptosis in HRECs Treated with High Glucose**

Apoptosis in HRECs was triggered beginning at 24-hour high-glucose treatment (Fig. 5). The proportion of early apoptosis, represented by AV−PI− cells, was significantly increased in a time-dependent manner, with 9.63% ± 1.26% at 24 hours and 12.90% ± 1.43% at 48 hours compared with controls (1.35% ± 0.68%). The proportion of late apoptosis, represented by AV+PI+ cells, was also significantly increased with 16.4% ± 1.24% at 24 hours and 19.73% ± 1.43% at 48 hours compared with controls (3.65% ± 0.92%).
DISCUSSION

Apoptosis and the expression of adhesion molecules in retinal vessels leading to leakage and occlusion of capillaries are two major early pathologic changes in diabetic retinopathy. Oxidative stress, involved in mitochondrial dysfunction and apoptosis in early damage of diabetic vascular complications, was demonstrated by recent studies.7,9 The overproduction of ROS seems to be the first and key event in the activation of other pathways involved in the pathogenesis of diabetic complications, including elevated polyol pathway activity, nonenzymatic glycation, and protein kinase C level.3,23–26 In agreement with other reports of nonendothelial cells exposed to high glucose,27,28 our results with HRECs also showed the overproduction of ROS and apoptosis, with high-glucose treatment associated with very early mtDNA damage 3 hours after treatment.

Some research has indicated that ROS, accompanied by increased NO generation, favors the formation of the strong oxidant peroxynitrite.29 Peroxynitrite, a potent initiator of DNA single-strand breakage, activates the nuclear enzyme poly(ADP-ribose) polymerase, which could lead to cell death.30 In addition, peroxynitrite by itself can increase the expression of adhesion molecules.31 Thus, apoptosis and increased expression of adhesion molecules are both closely related to ROS. With the pathways by which ROS leads to cell dysfunction elucidated, the mechanism of ROS overproduction in hyperglycemia needs further research.

Given the process of mtDNA damage leading to ROS overproduction and further mtDNA damage, mtDNA oxidative damage may be the early damage event in diabetic retinopathy. We found that mtDNA oxidative damage, as seen with 8-oxo-G lesion analysis, appeared very early in HRECs, 3 hours after exposure to high glucose, much earlier than the overproduction of ROS. Recently, the marked alteration of various protein subunits related to oxidative stress was observed on short-term, high-glucose treatment of nonendothelial cells or animals; the shortest effect time was 1 hour.32–34 Thus, the original mtDNA oxidative damage was not caused by the overproduced ROS in our high glucose–treated HRECs. mtDNA oxidative damage could have occurred quickly at first, contributing to the overproduction of ROS, and the overproduced ROS could have attacked mtDNA in return, which may explain why the Southern hybridization intensity with analysis of mtDNA damage was weak at 48 hours after exposure to high glucose.

A sensitive biomarker of oxidative DNA damage,35 8-oxo-G was quantitated with ELISA or quantitative PCR in other studies.36,37 The level of damage with 8-oxo-G lesions was higher and more extensive in mtDNA than in nuclear DNA in multiple tissues.18 Moreover, almost all mtDNA encodes respiratory chain subunits. Rapid mtDNA oxidative damage with high glucose might have affected transcription and translation of respiratory chain subunits, so we examined high glucose–induced alteration in the expression of several subunits. Compared with the expression of ATPsynβ, encoded by nuclear DNA, that of NADPH-1 and CO1, encoded by mtDNA, was downregulated. The subunits of complex I (NADPH-I) and complex IV (CO1) in the mitochondrial respiratory chain are the sites of ROS production and are involved in ROS regulation. Thus, oxidative mtDNA damage, changing the transcription of respiratory chain subunits, led to the overproduction of ROS in high glucose–treated HRECs.

A highly sensitive indicator of the energetic state of cells, Δψm was affected in various oxidative stress models.37–39 The
depolarization of mitochondria, followed by a permeable transmembrane pore opening and outflow of cytochrome c, predicts the activation of early apoptosis through the mitochondrial pathway. 10–12 We also observed a decrease in membrane potential at 12 hours and apoptosis at 24 hours after exposure to high glucose. mtDNA oxidative damage may promote the mitochondrial pathway of apoptosis under high glucose. Moreover, repairing mtDNA oxidative damage to prevent apoptosis has been confirmed in other models of oxidative stress. 13

The present study demonstrates that mtDNA oxidative damage acts as the trigger in vascular damage of diabetic retinopathy by setting in motion the vicious circle of mtDNA oxidative damage, leading to the overproduction of ROS and to further mtDNA damage. Our results also help in understanding the hyperglycemia “memory” found in in vivo or in vitro studies 13,14, once mtDNA oxidative damage occurs, though hyperglycemia is normalized, the cycle is switched on, continuously overproducing ROS involved in various downstream impairment pathways. This trigger may be the key to understanding the pathogenesis and treatment of diabetic retinopathy. However, elucidating the reason for this rapid mtDNA oxidative damage after short-term exposure to high glucose in HRECs requires further study.

Acknowledgments

The authors thank the Zhongshan Ophthalmic Center Eye Bank for its support.

References