Altered Mitochondrial Dynamics Contributes to Endothelial Dysfunction in Diabetes Mellitus

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Background—Endothelial dysfunction contributes to the development of atherosclerosis in patients with diabetes mellitus, but the mechanisms of endothelial dysfunction in this setting are incompletely understood. Recent studies have shown altered mitochondrial dynamics in diabetes mellitus with increased mitochondrial fission and production of reactive oxygen species. We investigated the contribution of altered dynamics to endothelial dysfunction in diabetes mellitus.

Methods and Results—We observed mitochondrial fragmentation (P=0.002) and increased expression of fission-1 protein (Fis1; P<0.0001) in venous endothelial cells freshly isolated from patients with diabetes mellitus (n=10) compared with healthy control subjects (n=9). In cultured human aortic endothelial cells exposed to 30 mmol/L glucose, we observed a similar loss of mitochondrial networks and increased expression of Fis1 and dynamin-related protein-1 (Drp1), proteins required for mitochondrial fission. Altered mitochondrial dynamics was associated with increased mitochondrial reactive oxygen species production and a marked impairment of agonist-stimulated activation of endothelial nitric oxide synthase and cGMP production. Silencing Fis1 or Drp1 expression with siRNA blunted high glucose–induced alterations in mitochondrial networks, reactive oxygen species production, endothelial nitric oxide synthase activation, and cGMP production. An intracellular reactive oxygen species scavenger provided no additional benefit, suggesting that increased mitochondrial fission may impair endothelial function via increased reactive oxygen species.

Conclusion—These findings implicate increased mitochondrial fission as a contributing mechanism for endothelial dysfunction in diabetic states. (Circulation. 2011;124:444-453.)

Key Words: diabetes mellitus, type 2 ■ endothelium ■ mitochondria ■ reactive oxygen species

Type 2 diabetes mellitus is an increasingly prevalent risk factor for atherosclerotic cardiovascular disease.1 A key mechanism in atherogenesis is endothelial dysfunction, which is characterized by decreased nitric oxide bioavailability and the development of an inflammatory phenotype that promotes atherosclerosis.2 An improved understanding of the mechanisms of endothelial dysfunction could stimulate new approaches for the prevention and management of diabetic cardiovascular disease.

Clinical Perspective on p 453

Prior studies implicate increased oxidative stress as a primary mechanism of endothelial dysfunction in diabetes mellitus.3 Exposure of cultured endothelial cells or isolated arterial tissue to high glucose concentrations increases the production of reactive oxygen species (ROS), which decrease nitric oxide bioavailability and increase expression of proinflammatory genes.2,3 Several enzymatic sources of ROS have been implicated in diabetes mellitus, including NADPH oxidase, aldose reductase, and components of the mitochondrial electron transport chain.4–7

There is growing appreciation of the importance of altered mitochondrial dynamics in diabetes mellitus.8 Mitochondria undergo cycles of fusion to form networks and fission to form smaller individual mitochondria.8,9 Proteins controlling fusion include mitofusin (Mfn)-1, Mfn2, and optic atrophy-1 (Opa1). Fission is regulated by dynamin-related protein-1 (Drp1) and fission-1 (Fis1). Fusion may
be beneficial by allowing the distribution of metabolites, proteins, and DNA throughout the network. At the end of their life cycle, dysfunctional mitochondria and damaged mitochondrial components are eliminated by fission and subsequent autophagy. Under pathological conditions, including diabetes mellitus, fission is increased and autophagy is impaired, leading to a loss of mitochondrial networks, accumulation of small dysfunctional mitochondria, and increased mitochondrial ROS.

Prior studies have demonstrated a loss of mitochondrial networks under hyperglycemic conditions in a variety of cell types, including islet cells, hepatocytes, skeletal muscle cells, circulating blood mononuclear cells, and endothelial cells. The functional consequences of altered mitochondrial dynamics in diabetes mellitus, however, remain incompletely understood in the human vasculature. The present study was designed to investigate the contribution of altered mitochondrial dynamics to increased ROS production and impaired nitric oxide bioavailability under diabetic conditions.

Methods

Study Subjects

Adult patients with type 2 diabetes mellitus and healthy volunteers were recruited at Boston Medical Center by advertisement. Diabetes mellitus was defined as fasting glucose ≥126 mg/dL or ongoing treatment for type 2 diabetes mellitus. Healthy volunteers were taking no medications; had blood pressure <140/90 mm Hg, fasting LDL cholesterol <160 mg/dL, and fasting glucose <100 mg/dL; and had never smoked or had stopped smoking for >1 year before enrollment. Fasting glucose and lipids were measured in the Boston Medical Center Clinical Laboratory. The study protocol was approved by the Boston Medical Center Institutional Review Board, and all participants provided written informed consent.

Noninvasive Vascular Function Testing

Brachial artery flow-mediated dilation was measured in each patient as previously described. Briefly, Doppler flow signals and 2-dimensional images were recorded from the brachial artery before and 1 minute after induction of reactive hyperemia by 5-minute cuff occlusion of the upper arm. We simultaneously measured endothelial vasodilator function in fingertip vessels using digital pulse amplitude tonometry (Endo-PAT, Itamar Medical Ltd, Caesarea, Israel).

Mitochondrial Reactive Oxygen Species Production

Mitochondrial ROS production was measured with fluorescence microscopy in live cells. The HAE CCs were incubated with 5 μmol/L MitoSox and 100 nmol/L MitoTracker Green FM (Invitrogen) for 30 minutes at 37°C and imaged according to the manufacturer’s instructions (excitation/emission, 510/580 nm). Colocalization of MitoSox and MitoTracker Green signal verified

Endothelial Cell Culture

Human aortic endothelial cells (HAECs) were purchased from Lonza, Inc and maintained with the EGM-2 Bullet Kit media (Lonza, Inc) containing 5 mmol/L glucose. Cells were cultured according to the manufacturer’s instructions at 37°C with 5% CO2. For immunofluorescence experiments, cells were grown in Falcon 4-well chambered slides (BD Biosciences). The effects of elevated glucose on endothelial cell function were investigated by incubating cells in EGM-2 media with a final glucose concentration of 30 mmol/L (540 mg/dL). This concentration is relevant to diabetic patients with severe hyperglycemia and has been used in many prior studies of the vascular effects of high glucose concentrations. To control for the osmotic effects of high glucose, we also completed studies using EGM-2 medium containing 5 mmol/L glucose and 25 mmol/L mannitol. Cells were stored at −80°C until immunofluorescence, protein, or gene expression measurements were performed.

Immunofluorescence Staining and Fluorescence Microscopy

Fixed samples were rehydrated with PBS containing 50 mmol/L glycine and permeabilized with 0.1% Triton X-100. After non-specific binding sites were blocked with 0.5% BSA, slides were incubated with two of the following primary antibodies: anti–von Willebrand factor 1:200, anti–cytochrome c oxidase-IV 1:300, anti–cytochrome c oxidase-IV 1:400, anti-Fis1 1:100, or anti–phosphorylated endothelial nitric oxide synthase (eNOS) 1:200. Slides were incubated with corresponding Alexa Fluor-488 and Alexa Fluor-594 secondary antibodies and mounted with Vectashield containing the nuclear stain DAPI (Vector Laboratories). Staining was performed in 1 batch to avoid any day-to-day variability in staining sessions.

Slides were examined with a fluorescence microscope (Nikon Eclipse TE2000-E) at ×100 magnification, and cellular images were digitally captured by a Photometric CoolSnap HQ2 Camera (Photometrics, Tucson, AZ). All images were captured at the same exposure time, and presented values are corrected for local background fluorescence. For the freshly isolated patient samples, endothelial cells were distinguished from other cells by von Willebrand factor staining. Images were captured and intensity was measured with NIS Elements AR Software (Nikon Instruments Inc, Melville, NY).

Assessment of Mitochondrial Networks

We used a semiquantitative scale to rate the extent of mitochondrial networks in patient cells and in HAECs under different experimental conditions. Cells were stained for cytochrome c and evaluated by 2 blinded observers. Mitochondrial network extent was graded on a scale from 0 to 3 (0 = wholly fragmented or punctuated mitochondria, 3 = clearly defined mitochondrial networks). The average score from 20 cells from each patient or condition was determined by 2 people and read separately, and the readings were averaged. The coefficient of variation was 7.5% (n=20 cells, measured 3 times) for repeated assessment of cells under the same experimental condition.

We also performed live cell imaging to further evaluate mitochondrial morphology. For these experiments, mitochondria were labeled with 100 nmol/L MitoTracker Green FM (Invitrogen) in HEPES-buffered physiological salt solution in accordance with the manufacturer’s recommendations. Images were captured at ×100 magnification.
that the ROS signal was localized to the mitochondria. Thirty cells were analyzed per experiment to obtain an average intensity value. In some experiments, the contribution of intracellular ROS to vascular dysfunction was evaluated by treating cells with a superoxide scavenger, tiron (5 mmol/L). The coefficient of variation for ROS measurement was 9.7%.

**RNA Isolation and Quantitative Gene Expression**

Total RNA was isolated from HAECs with the miRNeasy Mini kit (Qiagen, Inc) according to the manufacturer’s instructions. RNA was reverse transcribed to cDNA with TaqMan Reverse Transcription Reagents followed by cDNA preamplification with TaqMan PreAmp Master Mix (Applied Biosystems) according to the manufacturer’s instructions. Quantitative real-time polymerase chain reaction was performed with Taqman Gene Expression Assays (see the online-only Data Supplement) and the following protocol: 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. With GAPDH used as a loading control, results were interpreted by the relative quantity method (ΔΔCT).

**Protein Isolation**

Collected cell pellets were resuspended and briefly sonicated in a cell lysis buffer (Cell Signaling) containing 1% protease inhibitor cocktail (Sigma). Cell lysates were spun at 80°C before

**Western Blot Analysis**

Proteins were subjected to 8% to 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes (GE Healthcare). Membranes were initially blocked (PBS, 0.1% Tween 20, 5% nonfat dry milk) for 1 hour. Membranes were probed in blocking buffer containing one of the following primary antibodies: anti-Opa1, anti-Drp1, anti-Mfn2, or anti-Fis1 antibody, followed by the appropriate horse-radish peroxidase–conjugated secondary antibody. Immunoreactions were visualized with Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare). Membranes were stripped (62.5 mmol/L Tris-HCl [pH 6.8], 100 mmol/L β-mercaptoethanol, 2% SDS) for 30 minutes at 50°C and reprobed with anti-actin antibodies to verify equal protein loading. Resulting bands were quantified by densitometry.

**Assessment of Endothelial Nitric Oxide Synthase Activation and Endothelial cGMP Production**

Activation of eNOS was assessed by measuring phosphorylation of eNOS at serine 1177. After exposure of HAECs to 5 or 30 mmol/L glucose for 24 hours, cells were incubated with the calcium ionophore A23187 (1 μmol/L), acetylcholine (1 μmol/L), insulin (10 mmol/L), or vehicle for 5 minutes. Cells were then fixed and stained for phosphorylated eNOS as described above.

Bioactivity of endothelium-derived nitric oxide was assayed as change in cGMP concentration as previously described. Briefly, cells were equilibrated for 30 minutes with 200 μmol/L 3-isobutyl-1-methylxanthine to inhibit phosphodiesterases. Cells were then exposed to A23187 or vehicle for 5 minutes. Cells were lysed, and cell supernatants and protein pellets were stored at −80°C before processing. Supernatant cGMP was determined with a commercially available enzyme immunoassay kit (Cayman Chemical) and corrected for total cell protein.

**Suppression of Gene Expression With siRNA**

The HAECs were transfected with double-stranded siRNA targeting Fis1 or Drp1 using siPORT NeoFX Transfection Agent (Applied Biosystems) according to the manufacturer’s instructions. Forty-eight hours after transfection, cells were incubated with medium containing 5 or 30 mmol/L glucose for 24 hours. Cells were then processed as described above for assessment of mitochondrial network extent, protein expression, gene expression, or cGMP production. Nonspecific scrambled siRNA and GAPDH siRNA were used as negative and positive transfection controls, respectively.

**Statistical Analyses**

Statistical analyses were completed with Sigma Stat version 3.1 (Systat Software, Inc). Clinical characteristics and vascular function in the diabetic and healthy control groups were compared by use of the unpaired t test or χ² test for continuous and categorical variables, respectively. For cell experiments, we used the Student t test for 2-group comparisons. For experiments involving >2 groups, we used ANOVA with Student-Newman-Keuls multiple pairwise comparisons. Values of P<0.05 were considered statistically significant. Data are expressed as mean±SEM.

**Results**

**Study Subjects and Vascular Function**

We enrolled 10 patients with diabetes mellitus and 9 control subjects. The clinical characteristics and measures of vascular function are shown in the Table. As expected, the patients with diabetes mellitus had higher fasting glucose levels, higher body mass index, and lower high-density lipoprotein levels. Endothelial-dependent flow-mediated dilation of the brachial artery and flow-induced changes in pulse amplitude in the fingertip were lower in the diabetics compared with the control subjects. Baseline diameter, baseline flow, and extent of reactive hyperemic flow volume were similar in the 2 groups, suggesting that impaired flow-mediated dilation was not attributable to differences in arterial geometry or the stimulus for dilation.

**Mitochondrial Network Extent in Freshly Isolated Endothelial Cells**

Figure 1A shows mitochondrial morphology in freshly isolated endothelial cells. Cells from the healthy control
subjects displayed elongated, thread-like mitochondria in complex networks, whereas cells from diabetics displayed smaller punctate mitochondria. Group data show that patients with diabetes mellitus had lower mitochondrial network extent compared with control subjects (0.77 ± 0.09 versus 1.2 ± 0.06, respectively; \( P = 0.002 \)) as judged by blinded assessment of cytochrome c–stained cells (arbitrary scale, 0 to 3). Figure 1B shows Fis1 protein levels in the freshly isolated endothelial cells. Diabetic individuals have significantly higher Fis1 expression compared with healthy control subjects (1154 ± 60 versus 568 ± 68, respectively; \( P = 0.0001 \)). These results suggest altered mitochondrial dynamics in endothelial cells in patients with diabetes mellitus.

### Effect of High Glucose on Mitochondrial Networks and Dynamic Proteins in Human Aortic Endothelial Cells

To investigate potential mechanisms and functional consequences of altered mitochondrial dynamics in the endothelium, we exposed cultured HAECs to 30 mmol/L glucose as a model of hyperglycemic conditions. As shown in Figure 2A, exposure to high glucose induced a marked and sustained loss of mitochondrial networks over 24 hours. In separate experiments, exposure to 5 mmol/L glucose plus 25 mmol/L mannitol for 24 hours had no effect on network extent, suggesting that the effect of glucose exposure is not attributable to an osmotic effect.

The observed change in mitochondrial morphology was accompanied by a shift in mitochondrial dynamics gene and protein expression favoring fission. Exposure to 30 mmol/L glucose increased message (Figure 2B) and protein (Figure 2C) levels of the fission protein Fis1. Drp1 protein expression was increased (\( P < 0.05 \)), and there was a trend for increased Drp1 message (\( P = 0.07 \)). There was a modest increase in message level (\( P < 0.05 \)) but no significant change in protein level of the fusion protein Mfn2 (\( P = 0.11 \)). There were no significant changes in message or protein expression for the fusion proteins Opa1 and Mfn1.

### Effect of High Glucose on Mitochondrial Reactive Oxygen Species Production, Endothelial Nitric Oxide Synthase Activation, and Nitric Oxide Production

We examined the effects of high glucose concentration on mitochondrial ROS production in HAECs. As shown in Figure 3, high glucose concentrations induced a marked increase in MitoSox fluorescence that colocalized with MitoTracker Green fluorescence, consistent with a mitochondrial source of ROS.

We next examined the functional consequences of high glucose concentrations on eNOS activation and production of bioactive nitric oxide (Figure 4A through 4C). In cells exposed to 5 mmol/L glucose, acetylcholine, insulin, and calcium ionophore (A23187) individually activated eNOS, as reflected by an increase in eNOS phosphorylation at serine 1177. Exposure to 30 mmol/L glucose markedly impaired eNOS phosphorylation in response to all 3 agonists (\( P < 0.001 \) by ANOVA), consistent with impaired eNOS activation vasodilator function.

Calcium ionophore increased cGMP levels in endothelial cells exposed to 5 mmol/L glucose (Figure 4D), consistent with production of bioactive nitric oxide, which is capable of activating guanylyl cyclase. High glucose
exposure blunted the cGMP response to A23187, consistent with impaired bioactivity of endothelium-derived nitric oxide (P<0.001 by ANOVA).

Silencing Fis1 or Drp1 Protects Mitochondrial Networks and Blunts Reactive Oxygen Species Production

We next sought to determine whether increased mitochondrial fission contributes to altered mitochondrial morphology and ROS production in the presence of high glucose. To test this hypothesis, we used siRNA to silence the expression of the fission protein Fis1 or Drp1 in cultured endothelial cells. As shown in Figure 5, message levels were markedly reduced 48 hours after transfection with siRNA targeting Fis1 or Drp1, and there were corresponding decreases in protein expression.

As shown in Figure 6A, silencing Fis1 or Drp1 prevented the loss of mitochondrial networks after exposure of endothelial cells to 30 mmol/L glucose. Fis1 or Drp1 siRNA treatment did not alter mitochondrial network extent in cells exposed to 5 mmol/L glucose. As shown in Figure 6B, silencing either of these fission proteins markedly reduced ROS production in endothelial cells exposed to 30 mmol/L glucose. There was no effect of Fis1 or Drp1 siRNA treatment on ROS production in cells exposed to 5 mmol/L glucose.

Silencing Fis1 or Drp1 Prevents Glucose-Induced Impairment of Endothelial Nitric Oxide Synthase Activation

The effects of Fis1 or Drp1 siRNA on eNOS activation are displayed in Figure 7. As shown, silencing these fission proteins did not alter the basal expression of phosphorylated eNOS. However, silencing Fis1 or Drp1 expression prevented glucose-induced impairment of eNOS phosphorylation in response to calcium ionophore, acetylcholine, and insulin. As shown in Figure 8, silencing Fis1 expression also prevented glucose-induced impairment of agonist-induced cGMP production in endothelial cells. Collectively, these results suggest that glucose-induced alterations in mitochondrial dynamics have broad effects on endothelial function.

Decreased Reactive Oxygen Species May Account for the Protective Effects of Fis1 and Drp1 siRNA

We tested the hypothesis that reversal of endothelial dysfunction by inhibition of mitochondrial fission under high glucose conditions might be explained by the observed decreased in ROS. Phosphorylation of eNOS (Figure 7) and cGMP production (Figure 8) in response to calcium ionophore were preserved in the presence of 30 mmol/L glucose when cells were incubated with the
cell-permeable ROS scavenger tiron. A similar effect was produced by Fis1 or Drp1 siRNA. When combined with siRNA treatment, tiron had no additional benefit beyond the effect of Fis1 or Drp1 siRNA. These results are consistent with the possibility that glucose-induced alterations in mitochondrial dynamics affect eNOS activation and nitric oxide bioavailability via excess ROS.

**Discussion**

In this study, we observed altered mitochondrial morphology, reduced network extent, and increased Fis1 protein expression in endothelial cells from patients with diabetes mellitus with endothelial dysfunction compared with healthy volunteers. In cultured cells exposed to high glucose, we observed a similar loss of mitochondrial

![Figure 3](image-url)

**Figure 3.** High glucose concentration induces mitochondrial reactive oxygen species (ROS) production. As described in Methods, human aortic endothelial cells were incubated with 5 or 30 mmol/L glucose for 24 hours. Mitochondrial ROS production was assessed with 5 μmol/L MitoSox (red fluorescence), and mitochondria were localized with 100 nmol/L MitoTracker Green (green fluorescence). A, Representative fluorescence images showing increased ROS production that colocalizes with mitochondria in endothelial cells incubated with high glucose. High glucose is also associated with mitochondrial fragmentation. B, Pooled data showing that ROS production is higher in cells exposed to 30 mmol/L glucose. Data are shown as mean ± SEM for 3 experiments (*P < 0.01 vs 5 mmol/L glucose).

![Figure 4](image-url)

**Figure 4.** Elevated glucose level impairs endothelial nitric oxide synthase (eNOS) activation. Human aortic endothelial cells were incubated with 5 or 30 mmol/L glucose for 24 hours, and eNOS activation was evaluated as eNOS phosphorylation (p-ENOS) in response to acetylcholine (A), insulin (B), or A23187 (C), as described in Methods. As shown, 30 mmol/L glucose blunted the eNOS phosphorylation to each agonist (*P < 0.001 for interaction by ANOVA). Data are mean ± SEM for 3 experiments. D, Production of bioactive nitric oxide was assessed as the relative increase in cGMP in response to A23187. As shown, 30 mmol/L glucose blunted cGMP production (*P < 0.001 for interaction by ANOVA; *P < 0.01 vs control and vs 30 mmol/L glucose by Student-Newman-Keuls pairwise comparison). Data are mean ± SEM for 12 experiments.
networks accompanied by increased expression of fission proteins, and the effects were not attributable to an osmotic effect of glucose. The observed alteration in mitochondrial dynamics was associated with increased mitochondrial ROS production and a generalized impairment in agonist-stimulated eNOS activation and nitric oxide bioavailability. Silencing Fis1 or Drp1 expression blunted glucose-induced alterations in mitochondrial networks, ROS production, eNOS activation, and nitric oxide bioavailability. An intracellular ROS scavenger provided no additional benefit, suggesting that increased mitochondrial fission may impair endothelial function via increased ROS. These findings implicate altered mitochondrial dynamics as a contributing mechanism for endothelial dysfunction in diabetic states.

Several prior studies have demonstrated altered mitochondrial dynamics in nonvascular tissue from experimental models of diabetes mellitus and patients with diabetes mellitus or insulin resistance. For example, exposure of rat hepatocytes to high glucose concentrations induces mitochondrial fragmentation and increases ROS production, and the effect can be inhibited with dominant-negative Drp1. Similar findings are observed in pancreatic islet cells exposed to high glucose or free fatty acids that can be prevented by Fis1 siRNA. Mitochondrial fragmentation and decreased expression of Mfn2 are also observed in the skeletal muscle of obese insulin-resistant rats and diabetic humans. Recently, we observed fragmented mitochondria and increased ROS production in peripheral blood mononuclear cells from patients with type 2 diabetes mellitus.

Recent studies have shown that diabetic conditions alter mitochondrial dynamics and morphology in endothelial cells. Mitochondrial fragmentation occurs when cells from an immortalized endothelial cell line (EAhy926) are exposed to high glucose. Incubation of rat retinal endothelial cells with high glucose concentrations induces a loss of mitochondrial networks and increases apoptosis, which could contribute to diabetic retinopathy. In a recent study by Makino and colleagues, endothelial cells isolated from the coronary arteries of diabetic mice displayed fragmented mitochondria and increased ROS production. Those changes were associated with increased expression of Drp1 and decreased expression of Opa1, consistent with a shift toward mitochondrial fission. Drp1 siRNA prevented mitochondrial fragmentation under high glucose conditions, implicating increased fission as a cause of mitochondrial fragmentation.

The present study extends our understanding of the functional importance and clinical relevance of altered mitochondrial dynamics in the diabetic endothelium. We observed increased expression of Fis1 and a protective effect of Fis1 siRNA on mitochondrial networks in cultured human cells exposed to high glucose. We gained information about the clinical relevance of these mechanisms by showing a loss of mitochondrial networks in endothelial cells freshly isolated from diabetic patients that displayed impaired endothelium-dependent dilation. Most important, our study provides information about the functional consequences of altered mitochondrial dynamics in the diabetic endothelium by showing that increased mitochondrial ROS, impaired eNOS activation, and loss of nitric oxide bioavailability can be prevented by inhibiting mitochondrial fission.

Our study provides insight into the importance of mitochondrial network formation as a regulator of mitochondrial ROS generation in the endothelium. High glucose concentrations drive the electron transport chain to hyperpolarize the mitochondrial membrane and increase ROS production at complexes I and III via uncoupled respiration. In hepatocytes, mild membrane depolarization prevents ROS generation but does not prevent glucose-induced mitochondrial fragmentation, suggesting that increased mitochondrial ROS is a consequence of mitochondrial fragmentation, not the cause. Consistent with this prior work, we observed that inhibiting Fis1 or Drp1 expression was sufficient to completely inhibit glucose-induced ROS production and network fragmentation in endothelial cells. Collectively, these findings support the idea that network formation limits mitochondrial ROS production under conditions of increased fuel, possibly by allowing appropriate distribution of mitochondrial components, including uncoupling proteins and antioxidant enzymes. It also has been argued the fragmentation alters the spatial orientation of the electron transport chain enzymes in a manner that promotes uncoupled respiration...
and ROS production. On the other hand, Makino and colleagues showed that scavenging ROS prevents glucose-induced mitochondrial fragmentation in mouse endothelial cells, suggesting that ROS is a trigger for fission under these conditions. Further studies are needed to elucidate how high glucose concentrations stimulate mitochondrial fission and whether ROS is a cause, consequence, or exacerbating mechanism.

The present study suggests that mitochondrial fission is a major cause of endothelial dysfunction in the setting of hyperglycemia, likely via increased mitochondrial ROS. In addition to directly reacting with nitric oxide, ROS may lead to the uncoupling of eNOS, oxidation of cofactors, and oxidative modification of target enzymes. Another consequence of increased oxidative stress in the setting of high glucose may be O-linked N-acetylglycosylation of serine 1177 on eNOS, which blocks phosphorylation at this site and attenuates nitric oxide production. In our study, inhibiting the expression of fission proteins was sufficient to normalize agonist-induced eNOS phosphorylation and production of bioactive nitric oxide. The beneficial effects were observed when eNOS was activated by insulin, acetylcholine, or calcium ionophore, suggesting that the effect did not depend on the signaling mechanism for eNOS activation. Further studies are needed to determine whether there is a relation between mitochondrial dynamics and other enzymatic sources of ROS such as NADPH oxidase, which also has been implicated in endothelial dysfunction in diabetes mellitus and has been shown to be localized to mitochondria.

Our study has a number of limitations. First, the sample size for the clinical study was relatively modest, which precluded multivariable analysis to adjust for confounding factors. The analyses were completed in venous rather than arterial cells, reducing the relevance to atherosclerosis, although diabetes mellitus is a systemic disease that affects the endothelium in both arteries and veins. In addition, a prior study demonstrated significant correlations between findings in venous and arterial endothelial cells. Second, we may have overestimated the effects of high glucose on absolute mitochondrial ROS production by using an excitation wavelength of 510 nm as recommended by the manufacturer rather than 395 nm for the MitoSox experiments as has been suggested by Robinson and colleagues, although our studies focused on the relative changes in ROS according to glucose concentration. Further studies are merited to define interactions between mitochondrial and nonmitochondrial sources of ROS under diabetic...
conditions. Finally, 24-hour glucose exposure does not fully mimic the endothelial pathology observed in patients who have had diabetes mellitus for many years, although our model is well accepted for the study of mechanisms of diabetic endothelial dysfunction. These limitations are balanced by the novel information about mitochondrial dynamics in endothelial cells from human subjects and evidence for a mechanistic link between mitochondrial fission and impaired eNOS activation.

Our study may have important clinical implications. A large body of work indicates that endothelial dysfunction contributes to the pathogenesis of atherosclerotic disease in diabetes mellitus, but the mechanisms of impaired nitric oxide bioavailability in this setting remain incompletely understood. Pharmacological inhibitors of mitochondrial fission are under development. Identification of increased mitochondrial fission as a mechanism of endothelial dysfunction in diabetes mellitus suggests that such drugs might have utility as therapy to prevent and manage diabetic vascular disease.

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**Disclosures**

None.

**References**

Mitochondrial Fission in Endothelial Cells

Type 2 diabetes mellitus is an increasingly prevalent risk factor for atherosclerotic cardiovascular disease, and dysfunction of the vascular endothelium contributes to the development of diabetic vascular disease. We studied a previously unrecognized mechanism of endothelial dysfunction in human diabetes mellitus and show that an alteration in mitochondrial homeostasis is important. In addition to serving as the primary source of ATP in the cell, mitochondria participate in many other cellular functions. In the past, mitochondrial were viewed as discrete oval organelles, but recent studies have shown that mitochondrial fuse to form complex networks within the cell and that these networks are important for normal mitochondrial function. We observed that endothelial cells collected from patients with diabetes mellitus show a loss of normal networks and marked fragmentation of mitochondria. These changes were accompanied by increased levels of Fis1, a protein that controls mitochondrial fission. When commercially available endothelial cells were exposed to high glucose concentrations in tissue culture, we observed a similar loss of mitochondrial networks, increased Fis1, and impaired endothelial function. When we prevent mitochondrial fragmentation by blocking expression of Fis1, we maintain normal mitochondrial networks and prevent endothelial dysfunction. This study suggests a new target for therapy in diabetes mellitus and raises the possibility that a drug that prevents mitochondrial fission might protect against the development of vascular disease in diabetic patients. Such drugs are currently under development.
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Supplementary Material

Materials and Reagents

Primary antibodies were purchased from the following: Anti-vWF (Dako, Carpinteria, CA, USA); anti-Cytochrome C, anti-Opa1, anti-Drp1 (BD Biosciences, San Diego, CA, USA); anti-COX IV, anti-Mfn2, anti-GAPDH (Abcam, Cambridge, MA, USA); anti-Mfn1 (Novus Biologicals, Littleton, CO, USA); anti-Fis1 (Imgenex, San Diego, CA, USA); and anti-phosphorylated eNOS (p-eNOS) (Millipore, Billerica, MA, USA). Secondary horseradish peroxidase (HRP) conjugated antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA) and Alexa Fluor-488 and Alexa Fluor-594 conjugated secondary antibodies were obtained from Invitrogen (Carlsbad, CA, USA). VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA), guanosine 3',5'-cyclic mononucleotide EIA kit (Cayman Chemical, Ann Arbor, Michigan, USA), Pierce BCA Protein Assay Reagent (Thermo Fisher Scientific, Rockford, IL, USA), Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA, USA). PVDF membrane and Amersham ECL Plus Western Blotting Reagents were purchased from GE Healthcare, Piscataway, NJ. Glass bottom dishes and plates were purchased from MatTek, Ashland, MA, USA. All siRNA and Taqman gene expression assays and siRNA assays were all obtained from Applied Biosystems (Carlsbad, CA, USA). All other reagents (IBMX, A23187, TCA, insulin, acetylcholine, poly-lysine, and protease inhibitor) were obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA).
**Gene Expression Assays**

FIS1 (Hs00211420_m1), DNL1 (Hs00247147_m1), OPA1 (Hs00323399_m1), MFN1 (Hs00966851_m1), MFN2 (Hs00208382_m1)

**siRNA Assays**

FIS1 siRNA ID: s27266, DRP1 siRNA ID: s19560, Select GAPDH Positive Control siRNA (4390849), Select Negative Control #1 siRNA (4390843)