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).

Materials

Please see the online-only Data Supplement for a list of the sources for materials and reagents for the endothelial cell experiments.

Fresh Isolation of Endothelial Cells From Study Subjects

Endothelial collection was performed as previously described. Briefly, local anesthesia was administered and an 18- or 20-gauge catheter was placed in an arm vein using sterile technique. Endothelial cells were collected by gently abrading the luminal surface of the vein with a 0.018-in J wire. For immunofluorescence experiments, endothelial cells were recovered from the wire with a dissociation buffer, fixed with 4% paraformaldehyde, and plated on poly-lysine-coated slides (Sigma). Fixed slides were stored at −80°C until further processing. For live cell studies, recovered endothelial cells were allowed to adhere to poly-lysine–coated glass bottom dishes (MatTek, Ashland, MA) for 6 hours in EGM-2 medium (Lonza Inc, Walkersville, MD) before imaging.

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 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.

Mitochondrial Reactive Oxygen Species Production

Mitochondrial ROS production was measured with fluorescence microscopy in live cells. The HAECs 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...
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 10 000 rpm for 10 minutes at 4°C, and the resulting supernatants were stored at −80°C. Protein concentration was determined by the Pierce BCA protein assay (Thermo Fisher Scientific).

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-Opal1, 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), or vehicle for 5 minutes. Cells were then fixed and stained for phosphorylated eNOS 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. Endothelium-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

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FMD, brachial artery flow-mediated dilation expressed as percentage change from baseline; and PAT ratio, pulse amplitude tonometry as described in Methods (Mean±SD).

Maximum percentage change from baseline; and PAT ratio, pulse amplitude tonometry as described in Methods (Mean±SD).
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 guanyl cyclase. High glucose...
exposure blunted the cGMP response to A23187, consistent with impaired bioactivity of endothelium-derived nitric oxide \( (P<0.001 \text{ by ANOVA}) \).

**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.** 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.

**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.
human insulin-resistant rats and diabetic
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-
duced 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 mito-
ochondrial dynamics in nonvascular tissue from experimen-
tal models of diabetes mellitus and patients with diabetes
mellitus or insulin resistance. For example, exposure of rat
hepatocytes to high glucose concentrations induces mito-
ochondrial fragmentation and increases ROS production,
and the effect can be inhibited with dominant-negative
Drp1.11 Similar findings are observed in pancreatic islet
cells exposed to high glucose or free fatty acids that can be
prevented by Fis1 siRNA.12 Mitochondrial fragmentation
and decreased expression of Mfn2 are also observed in the
skeletal muscle of obese insulin-resistant rats and diabetic
humans.14–25 Recently, we observed fragmented mitochon-
dria and increased ROS production in peripheral blood
mononuclear cells from patients with type 2 diabetes mellitus.16

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 ex-
posed to high glucose.17 Incubation of rat retinal endothe-
rial cells with high glucose concentrations induces a loss of
mitochondrial networks and increases apoptosis, which
could contribute to diabetic retinopathy.18 In a recent study
by Makino and colleagues,19 endothelial cells isolated
from the coronary arteries of diabetic mice displayed
fragmented mitochondria and increased ROS production.
These changes were associated with increased expression
of Drp1 and decreased expression of Opa1, consistent with
a shift toward mitochondrial fission. Drp1 siRNA pre-
vented 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 cul-
tured human cells exposed to high glucose. We gained
information about the clinical relevance of these mech-
anism 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 func-
tional consequences of altered mitochondrial dynamics in
the diabetic endothelium by showing that increased mito-
ochondrial 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 mito-
ochondrial ROS generation in the endothelium. High glu-
cose concentrations drive the electron transport chain to
hyperpolarize the mitochondrial membrane and increase
ROS production at complexes I and III via uncoupled respiration.7 In hepatocytes, mild membrane depolariz-
ination 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.11 Consistent
with this prior work, we observed that inhibiting Fis1 or
Drp1 expression was sufficient to completely inhibit
glucose-induced ROS production and network fragmenta-
tion in endothelial cells. Collectively, these findings sup-
port the idea that network formation limits mitochondrial
ROS production under conditions of increased fuel, possi-
bly by allowing appropriate distribution of mitochondrial
components, including uncoupling proteins and antioxi-
dant enzymes.8 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

![Figure 6](http://circ.ahajournals.org/)

Silencing fission-1 protein (Fis1) or dynamin-related protein-1 (Drp1) expression prevents glucose-induced loss of mitochondrial networks and glucose-induced reactive oxygen species (ROS) production. A. Mitochondria were imaged in human aortic endothelial cells (HAECs) with MitoTracker Green, and representative images show that silencing either fission protein maintained mitochondrial networks under high-glucose conditions. Pooled data show a protective effect on network extent (*P<0.001 for interaction by ANOVA). B. Mitochondrial ROS production was imaged in HAECs with MitoSox, and representative images show that silencing either fission protein produces a marked decrease in ROS production. Pooled data confirm that Fis1 and Drp1 siRNA prevent glucose-induced increases in ROS (*P<0.001 for interaction by ANOVA; *P<0.001 vs 5 mmol/L glucose; #P<0.01 vs control by Student-Newman-Keuls pairwise comparison). Data are mean ± SEM for 3 experiments.
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 mitochondrial 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)