Adverse Alterations in Mitochondrial Function Contribute to Type 2 Diabetes Mellitus–Induced Endothelial Dysfunction in Humans

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Objective—Mitochondrial dysfunction plays a key pathophysiological role in type 2 diabetes mellitus (T2DM)–related endothelial dysfunction. Data delineating relationships between mitochondrial and endothelial dysfunction in humans with T2DM are lacking.

Methods and Results—In 122 human subjects (60 with T2DM, 62 without T2DM), we measured endothelial function by brachial artery ultrasound (flow mediated dilation %) and digital pulse amplitude tonometry. Endothelial function in arterioles isolated from gluteal subcutaneous adipose was measured by videomicroscopy. In arterioles and mononuclear cells, we measured inner mitochondrial membrane potential (ΔΨm), mitochondrial mass, and mitochondrial superoxide production using fluorophores. Endothelial function was impaired in T2DM versus nondiabetes mellitus. ΔΨm magnitude was larger, and mitochondrial mass was lower in arterioles and mononuclear cells in T2DM. Mononuclear mitochondrial mass correlated with flow-mediated dilation % and pulse amplitude tonometry (r=0.38 and 0.33, P=0.001 and 0.02, respectively), and mononuclear mitochondrial superoxide production inversely correlated with flow-mediated dilation % (ρ=−0.58, P=0.03). Endothelial function was impaired in T2DM. Low doses of 2 different mitochondrial uncoupling agents (carbonyl cyanide m-chlorophenyl hydrazone and 2,4-dinitrophenol) that reduce ΔΨm magnitude and a mitochondrial-targeted antioxidant (MitoTEMPOL) improved endothelial function and reduced mitochondrial superoxide levels.

Conclusion—Mitochondrial dysfunction may play a central role in the origin and maintenance of endothelial dysfunction in T2DM.

Key Words: diabetes mellitus type 2 • endothelium • mitochondria • NO • oxidative stress

The worldwide incidence of type 2 diabetes mellitus (T2DM) is expected to exceed 350 million people in 2030, with the incidence rate of T2DM increasing in all segments of the population.1,2 The majority of morbidity and mortality related to diabetes mellitus is secondary to the macrovascular (myocardial infarctions, stroke) and microvascular (blindness, renal failure) complications of this disease.2,3 The pathogenesis of clinically overt vascular disease in T2DM, while not completely elucidated, seems to begin with the initial development of vascular endothelial dysfunction.4–8 Endothelial dysfunction encompasses the procoagulant, prothrombotic, and proinflammatory endothelial phenotype known to precede the development of clinically relevant vascular disease and portend future cardiovascular risk.9,10

The molecular origins of insulin-resistance–related vascular endothelial dysfunction include the induction of excessive oxidative stress7,11 and the activation of proinflammatory and procoagulant pathways involving nuclear factor-κB and protein kinase Cβ.12–14 Hyperglycemia and excess circulating free fatty acids seem to be responsible for the initiation of vascular oxidative stress and inflammation.15,16 Interestingly, in cell culture models, activation of these deleterious pathways also seems to require the development of mitochondrial dysfunction.17,18 Based on prior cell culture and animal work, insulin-resistance–related mitochondrial dysfunction is typified by 2 key alterations in the mitochondrial phenotype, leading to excessive mitochondrial reactive oxygen species (ROS) production: inner mitochondrial membrane hyperpolarization19,20 and reduced mitochondrial mass and mitochondrial network complexity.17,21 We recently observed such alterations in mitochondrial function and morphology in peripheral blood mononuclear cells and isolated venous endothelial cells from patients with T2DM.20,21

Excessive mitochondrial ROS production is known to cause deleterious vascular cell signaling and subsequent endothelial dysfunction.22 Despite cell and animal data supporting
a potential role for mitochondrial dysfunction in the origins of vascular dysfunction in T2DM, there are no data linking mitochondrial dysfunction to vascular endothelial dysfunction in intact human arteries and arterioles. We hypothesized that (1) systemic measurements of arterial endothelial function would correlate with systemic measurements of mitochondrial homeostasis derived from circulating mononuclear cells; (2) arterioles of patients with T2DM would display phenotypic mitochondrial dysfunction, with reduced mitochondrial mass and greater mitochondrial inner membrane polarization relative to nondiabetic humans; and (3) mitochondrial hyperpolarization and excessive mitochondrial superoxide production would relate mechanistically to arteriolar endothelial dysfunction in patients with T2DM.

**Methods**

**Subjects**

The study protocol and advertisements were approved by the Medical College of Wisconsin’s Institutional Research Board. The inclusion/exclusion criteria for nondiabetic subjects and T2DM subjects were previously described. A total of 122 individuals between 35 and 70 years of age were consecutively enrolled. We previously reported mitochondrial membrane potential, mass, and superoxide production in 59 of these subjects (27 subjects with T2DM, 32 with non-T2DM). For T2DM subjects, the additional cohort of 33 subjects had slightly higher systolic blood pressure (131±4 versus 125±8 mm Hg, P=0.04), heart rate (70±2 versus 64±5 bpm, P=0.06), and fasting triglycerides (151±4 versus 128±5, P=0.02) but otherwise did not differ with respect to other baseline characteristics as shown in the Table. There were no significant differences in the baseline characteristics of the additional 30 non-T2DM subjects. Subjects were defined as having T2DM based on standard criteria. Nondiabetic control subjects were excluded if they met criteria for metabolic syndrome, had a history of cardiovascular disease by history or documented myocardial infarction, had a major chronic illness, were pregnant at the time of screening, or had smoked within 1 year of enrollment. All subjects fasted for at least 6 hours before their study visits, and all studies began between 7:30 am and 9:00 am. Subjects on antihypertensive medications were asked to hold these medications for 24 hours before their study visits. Subjects on oral diabetes mellitus medications were instructed to hold their morning pills until completion of the study protocol. All subjects had blood drawn for measurements of fasting glucose levels, lipid profiles, measurements of insulin resistance, and measurements of mitochondrial homeostasis. Insulin resistance was estimated using both homeostasis model assessment-insulin resistance=1/[(fasting insulin μU/mL)/(fasting glucose mmol/L)][22.5 and the quantitative insulin-sensitivity check index=1/[(log(insulin)+log(glucose)].

**Materials**

Acetylcholine (Ach), 2,4 dinitrophenol (DNP)—reduces mitochondrial membrane potential magnitude, Δψm, carbonyl cyanide m-chlorophenyl hydrazone (CCCP)—also reduces Δψm magnitude, and L-Nω-nitroarginine methyl ester (L-NAME—NO synthase inhibitor) were purchased from Sigma-Aldrich (St. Louis, MO). Body mass index, kg/m² 34.6±8.0 28.2±5.6 <0.001 Smoking status, % (Previous) 37 31 0.48 Family history of CAD, % 37 20 0.03 Waist circumference, cm 111±17 94±16 <0.001 Systolic blood pressure, mm Hg 128±29 125±19 0.46 Diastolic blood pressure, mm Hg 72±10 73±12 0.29 Heart rate, beats/min 70±12 65±10 0.28 Total cholesterol, mg/dL 179±33 188±38 0.67 LDL cholesterol, mg/dL 98±30 114±30 0.01 HDL cholesterol, mg/dL 51±14 56±15 0.08 Triglycerides, mg/dL 152±73 95±36 <0.001 Glucose, mg/dL 134±52 82±11 <0.001 Insulin, μU/mL 20±11 13±8 <0.001 hs-CRP, mg/dL 4.1±4.4 2.6±3.5 0.04 HOMA-IR 7.0±5.2 2.9±2.1 <0.001 QUICKI 0.30±0.06 0.34±0.03 <0.001 Glycosylated hemoglobin, % 6.8±1.6 5.6±1.0 0.006

**Glycosylated hemoglobin** is used to assess both FMD% and NMD%. For FMD%, our intra- and interobserver correlation coefficients are 0.99 and 0.87, respectively. The mean absolute difference measured between observers is 1.0±0.8%.

**Digital Pulse Amplitude Tonometry**

Digital pulse amplitude was measured using a pulse amplitude tonometry (PAT) device placed on the index finger of each hand (Endo-PAT 2000; Itamar Medical, Caesarea, Israel). We measured and reported PAT as the natural log of the ratio of the baseline pulse amplitudes and postdeflation pulse amplitudes 90 to 120 seconds after cuff deflation as described previously. Greater detail on the methods for PAT can be found in the online-only Data Supplement.

**Measurement of In Vitro Endothelial Function**

Please see the online-only Data Supplement for full methodological details on the procedure used to obtain human gluteal adipose arteries and preparation of these vessels for study of endothelium-dependent and independent vasodilation by videomicroscopy. Our success rate for obtaining usable arteries for study by biopsy was 75%. As each biopsy yielded =1 usable arteriole, we could...
not perform all measurements on all vessels. The type of study done with each vessel was randomly selected and stratified by study group. Endothelium-dependent vasodilation was determined at increasing concentrations of Ach from 10^{-10} to 10^{-5} mol/L. The endothelial NO synthase dependence of vasodilation was determined by concomitant L-NAME exposure (1 mmol/L). To determine whether partial depolarization of the mitochondrial inner membrane altered endothelial function, vessels were additionally exposed (intraluminally) to either CCCP (100 nmol/L) or DNP (50 nmol/L) for 30 minutes before measurements of Ach-induced vasodilation (flow rate < 5 dynes/cm²). To determine whether lowering mitochondrial superoxide levels improved endothelial function, a subset of vessels were intraluminally exposed to MitoTEMPO (1 mmol/L) for 30 minutes before measuring Ach-induced vasodilation. All vessels were exposed to 0.2 mol/L papaverine at the end of each study to determine the level of endothelium-independent vasodilation.

Vascular Measurements of Mitochondrial Homeostasis

**NAO and JC-1 Arteriolar Endothelium Measurements**

Following dissection, cannulation, and equilibration, NAO (25 nmol/L), JC-1 (100 nmol/L), or TMRM (1 μmol/L) was allowed to flow through the vascular lumen for 30 minutes at a low rate (shear < 5 dynes/cm²). Before fluorophore exposure, arterioles to be exposed to NAO and TMRM were imaged by fluorescent microscopy to serve as negative controls. To verify the endothelial specificity of our studies with NAO and JC-1, we denuded several vessels with air boluses (3 for each fluorophore) before visualization and imaged vessel that were not exposed to fluorophores as well. Sample images and results confirming the endothelial specificity of these studies are shown in Figures I and II in the online-only Data Supplement. NAO and TMRM fluorescence intensities were measured by fluorescent microscopy (Eclipse TE 200; Nikon, Japan) with Ex/Em of 492/526 nm and 549/573 nm, respectively. Reported NAO and TMRM fluorescence intensities are the net of the NAO- or TMRM-exposed arteriole from each individual minus the individual’s matched control fluorescence intensity. Red and green JC-1 fluorescence were detected by inverted laser scanning confocal microscopy (Eclipse TE2000-U; Nikon, Tokyo, Japan) with Ex/Em of 488/520 nm and 578 nm. All fluorescence intensities in this study were analyzed using MetaMorph 6.1 (Universal Imaging, West Chester, PA).

**MitoSox and DAF2-DA Measurements on Arterioles From Subjects With and Without T2DM**

Arterioles were incubated in HEPES buffer under normal glucose conditions (5 mmol/L) with either MitoSox (5 μmol/L) or DAF2-DA (5 μmol/L) for 30 minutes. MitoSox fluorescence intensity was measured by fluorescent microscopy (Eclipse TE200; Nikon, Japan) at Ex/Em of 510/572 nm for MitoSox or 492/526 nm for DAF2-DA. Negative control images from the same vessels were taken before giving fluorescent dye. Reported MitoSox and DAF2-DA fluorescence intensities are the net of the MitoSox- or DAF2-DA-exposed arteriole from each individual minus the individual’s matched negative control vessel background fluorescence.

**Systemic Measurements of Mitochondrial Homeostasis and Their Associations With Systemic Endothelial Function**

In a subset of 71 of the 122 subjects (35 nondiabetic, 36 diabetic), we measured NAO fluorescence intensity in mononuclear cells. We measured mitochondrial membrane potential in a subset of 95 of the 122 subjects (46 nondiabetic, 49 diabetic). As we previously reported in a smaller set of these subjects, NAO fluorescence intensity was significantly lower in patients with T2DM than controls (235±19 versus 127±17 AU for nondiabetic subjects versus subjects with T2DM, P<0.001), and mitochondrial membrane potential was significantly more polarized in patients with T2DM compared to those without T2DM. Because of limitations on the amount of cells available from each patient for the studies presented, MitoSox ([n=13]) and JC-1 ([n=95]), and NAO ([n=71]) were only performed on a subset of the full study population.

**Statistical Analyses**

Baseline characteristics, measurements of mitochondrial homeostasis in both mononuclear cells and arterioles, and in vivo vascular endothelial function for nondiabetic subjects and subjects with T2DM were compared using unpaired t tests, Wilcoxon rank-sum tests, or χ² tests as appropriate. Correlations between mononuclear cell measurements of mitochondrial homeostasis and measures of vascular homeostasis were performed using Spearman’s ρ. For significant univariate correlations, stepwise multivariable linear regressions models were constructed to determine whether systemic measures of mitochondrial function predicted systemic measures of endothelial function using the following covariables: age, sex, systolic blood pressure, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, smoking status, family history of coronary artery disease, and fasting glucose. Dose-response curves within each subject group were compared by repeated measures ANOVA, and relevant between-group comparisons were made using 2-way mixed ANOVA analyses. Levels of mitochondrial superoxide and NO production were compared using mixed or repeated measures ANOVA as appropriate. DAF2-DA fluorescence data was log transformed for analyses to assure normal data distribution. Post hoc testing was applied to these analyses if significant differences were noted as appropriate. P<0.05 was considered significant.
T2DM subjects relative to nondiabetic subjects (13.1±0.8 versus 17.4±0.9 for nondiabetic subjects versus subjects with T2DM, \(P<0.001\)). We have also previously reported excess mitochondrial superoxide production in T2DM subjects relative to nondiabetic subjects in a subset of this population.\(^{20}\) Interestingly, we now found a strong negative correlation between paired measurements of mononuclear cell NAO fluorescence intensity and MitoSox fluorescence intensity (\(n=13, \rho=-0.72, P=0.006\); Figure III in the online-only Data Supplement).

Results of systemic measures of endothelial function in both T2DM and non-T2DM subjects are summarized in Table II in the online-only Data Supplement. FMD\% (6.2±1.6 versus 3.7%±1.7%, \(P<0.001\)) and ln PAT (0.74±0.61 versus 0.48±0.42, \(P=0.02\)) were significantly lower in patients with T2DM. There was a trend toward reduced nitroglycerin-mediated dilation in T2DM (NMD\%: 21.9%±7.0% versus 19.5%±6.3%, \(P=0.07\); NMD mm: 0.82±0.26 versus 0.73±0.21, \(P=0.05\)). We found no differences in baseline peak shear (42±15 versus 40±14 dynes/cm\(^2\) for non-T2DM and T2DM, respectively, \(P=0.36\)) or peak hyperemic shear (78±28 versus 71±23 dynes/cm\(^2\) for non-T2DM and T2DM, respectively, \(P=0.09\)) between groups.

We report significant univariate correlations between systemic measurements of endothelial function and monocyte mitochondrial homeostasis in Figure 1. NAO fluorescence intensity positively correlated with FMD\% (\(\beta=0.22\), \(P=0.36\)) and total cholesterol (\(\beta=−0.40\), \(P=0.047\)) and fasting glucose (\(\beta=−0.06\) and \(−0.29, P=0.001\) and 0.03 for FMD\% and ln PAT, respectively) remained independent predictors of FMD\% (model \(R^2=0.24, P<0.001\)) and ln PAT (model \(R^2=0.20, P=0.004\)). No single variable was an independent predictor of NMD\%. In a multivariable analysis for independent predictors of FMD\% including MitoSox fluorescence rather than NAO fluorescence, only MitoSox fluorescence (\(\beta=−0.73, P=0.003\)) and total cholesterol (\(\beta=−0.44, P=0.046\)) were independent predictors (model \(R^2=0.60, P=0.006\)).

**Mitochondrial Dysfunction in Arterioles From Patients With T2DM**

We measured mitochondrial mass, Δψ\(m\), and mitochondrial superoxide production in subcutaneous arterioles from random subsets of our study participants (Figure 2). Mitochondrial mass as measured by NAO fluorescence intensity was significantly lower in the arteriolar endothelium of subjects with T2DM (54±24 versus 199±58 AU for T2DM \([n=10]\) and nondiabetic subjects \([n=11]\), respectively; Figure 2A), whereas Δψ\(m\) was higher in the endothelium of subjects with T2DM as measured by JC-1 (2.6±0.08 versus 1.9±0.06 for T2DM \([n=5]\) and nondiabetic subjects \([n=9]\), respectively; Figure 2B). Additional arterioles exposed to TMRM confirmed our JC-1 findings (224±70 versus 56±21 AU for T2DM \([n=8]\) and nondiabetic subjects \([n=8]\), respectively). There were no significant correlations between JC-1 and MitoSox fluorescence and NMD\% (\(\rho=−0.10, −0.31, P=0.35, 0.31\), respectively) or ln PAT (\(\rho=−0.18, −0.29, P=0.13, 0.33\)). The correlation between JC-1 fluorescence and FMD\% was not significant (\(\rho=−0.06, P=0.59\)).

In stepwise multivariable linear regression models for FMD\% and ln PAT, only NAO fluorescence (\(\beta=0.22\) and 0.27, \(P=0.047\) and 0.045 for FMD\% and ln PAT, respectively) and fasting glucose (\(\beta=−0.40\) and \(−0.29, P=0.001\) and 0.03 for FMD\% and ln PAT, respectively) remained independent predictors of FMD\% (model \(R^2=0.24, P<0.001\)) and ln PAT (model \(R^2=0.20, P=0.004\)). No single variable was an independent predictor of NMD\%.

**Figure 1.** Correlations between systemic measures of endothelial function and mitochondrial homeostasis. Mitochondrial mass in human monocytes measured by acridine orange 10-nonyl bromide (NAO) fluorescence intensity was positively correlated with flow-mediated dilation (\(\rho=0.38, P=0.001, n=71\)) (A), digital pulse amplitude tonometry (PAT) (\(\rho=0.33, P=0.02, n=53\)) (B), and brachial artery nitroglycerin-mediated dilation (\(\rho=−0.27, P=0.001, n=62\)) (C). Mitochondrial superoxide production in human monocytes showed a strong negative correlation with flow-mediated dilation \% (FMD\%) (\(\rho=−0.58, P=0.03, n=14\)) (D). T2DM indicates type 2 diabetes mellitus.
respectively; Figure 2C). There was a strong trend toward increased mitochondria specific superoxide in arterioles from patients with T2DM (43±5 versus 26±7 AU for T2DM [n=10] and nondiabetic subjects [n=9], respectively; P=0.05 versus nondiabetic subjects; Figure 2D). In an additional 5 T2DM and 4 non-T2DM subjects, treatment of arterioles for 30 minutes with 100 nmol/L CCCP resulted in significant reductions in mitochondrial superoxide levels in both subjects with and without T2DM (Figure 2E). Representative images for this experiment are included in Figure IV in the online-only Data Supplement.

**Effect of Partial Mitochondrial Uncoupling and Mitochondrial Antioxidant Exposure on Human Arteriolar Endothelial Function in T2DM and Nondiabetic Subjects**

Subcutaneous arterioles isolated from patients with T2DM demonstrate impaired endothelium-dependent vasodilation compared with nondiabetic controls (P<0.001; Figure 3A). L-NAME completely inhibits Ach-induced endothelium-dependent vasodilation in these vessels in both T2DM patients and non-T2DM controls (Figure 3A). Thirty to sixty minutes of exposure of T2DM arterioles to 100 nmol/L of the mitochondrial uncoupler CCCP to reduce Δψm magnitude completely reversed endothelial dysfunction in T2DM arterioles (P=0.02; Figure 3B). Cotreatment with L-NAME abrogated the beneficial effect of CCCP (Figure 3C). A different uncoupling agent, DNP, also reversed endothelial dysfunction in T2DM vessels (Figure 3D). CCCP (P=0.94) and DNP had no effect on Ach-induced vasodilation of vessels from nondiabetic subjects. There were no significant differences in the Ach response in T2DM vessels exposed to CCCP compared with those exposed to DNP (P=0.21). No differences in papaverine responses were seen between T2DM and nondiabetic subjects, regardless of CCCP or DNP exposure state. In an additional 4 T2DM subjects, we found that NO bioavailability significantly increased in arterioles from T2DM vessels after exposure to 100 nm CCCP (P=0.03; Figure 4). Representative images for these experiments are included in Figure V in the online-only Data Supplement.
MitoTEMPOL, a mitochondrial-specific superoxide dismutase mimetic, significantly improved arteriolar endothelial function in vessels from a subset of T2DM subjects (n=7; Figure 5A). L-NAME significantly blunts the ameliorative effect of mitoTEMPOL (n=3; Figure 5B). Exposure to CCCP, DNP, or mitoTEMPOL has no significant effect on resting arteriolar diameters in any of the vasoactivity studies (Table III in the online-only Data Supplement).

Correlations between paired Ach vasodilation data and measurements of glycosylated hemoglobin (n=18: 10 T2DM, 8 non-T2DM) demonstrate a strong negative correlation ($\rho=-0.59$, $P=0.01$) between glycosylated hemoglobin levels and vasodilation to peak dose Ach (10$^{-4}$ mol/L). When stratified by T2DM status, the statistical significance of correlations is likely due to small numbers but the strong negative directionality remained ($\rho=-0.33$ and $-0.50$, $P=0.36$ and 0.20 for T2DM and non-T2DM, respectively). For the n=42 (17 T2DM, 22 non-T2DM) paired fasting plasma glucose and peak dose Ach-paired measurements, fasting glucose strongly negatively correlated with peak Ach dose vasodilation as well ($\rho=-0.49$, $P=0.001$). However, when stratified by T2DM status, these correlations were significantly weakened ($\rho=-0.10$ and $-0.08$, $P=0.67$ and 0.74 for T2DM and non-T2DM, respectively).

**Discussion**

Our studies reveal several substantial findings linking T2DM-associated mitochondrial dysfunction with human arterial endothelial dysfunction. In arterioles from subjects with T2DM, we observed mitochondrial hyperpolarization, reduced mitochondrial mass, and excessive mitochondrial superoxide production relative to nondiabetic controls. These alterations in mitochondrial function were associated with impaired endothelium-dependent vasodilation to Ach, which with limited data, seems to be most associated with glycemic control. Two different treatments that reduce $\Delta \psi_m$ magnitude as well as a mitochondrial-specific superoxide scavenger improved NO synthase–dependent endothelial function in arterioles from subjects with T2DM and improved NO bioavailability in these vessels. As we previously reported, these adverse alterations...
in mitochondrial function also occur in peripheral blood mononuclear cells, and we have expanded on our prior findings by showing that reduced mitochondrial mass and increased mitochondrial superoxide are associated with impaired systemic measurements of human endothelial function. Taken together, our data suggest a potential mechanistic role for mitochondrial dysfunction (Δψm hyperpolarization, reduced mitochondrial mass, and excessive mitochondrial ROS production) in the development and maintenance of endothelial dysfunction in patients with T2DM. Finally, our data support the concept that mitochondrial dysfunction in T2DM is systemic and that measurements made in easily obtainable circulating cells relate to noninvasive measures of endothelial vasomotor function.

Patients with T2DM exhibit both conduit vessel and microvascular endothelial dysfunction.4–8 Endothelial dysfunction in this population predates the development of T2DM in humans because hyperglycemia with and without prevalent insulin resistance can induce systemic endothelial dysfunction.10 Although the pathophysiological mechanisms of endothelial dysfunction in T2DM are yet to be fully elucidated, human cross-sectional studies examining other cell types strongly suggest that patients with T2DM have impaired mitochondrial function, with concomitant reductions in mitochondrial ATP production capacity and increased mitochondrial ROS production that predate their expression of phenotypical T2DM.20,29,30 Cell culture and animal work suggest that exposures to high glucose and free fatty acid levels impair endothelial function through induction of excessive mitochondrial oxidative stress.10,16,17,24 Our data effectively combine and extend the previous cross-sectional human studies and mechanistic cell and animal work by demonstrating (1) arterioles from T2DM patients have endothelial NO synthase–dependent endothelial dysfunction mechanistically related to mitochondrial ROS production and Δψm hyperpolarization; and (2) alterations in mitochondrial mass and ROS production are systemic and predictive of systemic in vivo measures of endothelial function.

Our data support the concept that Δψm is a central regulator of endothelial function in T2DM.24 Δψm hyperpolarization, occurring secondary to elevated glucose levels, increases ROS production from the mitochondrial electron transport chain because of reduced electron flux and a subsequent increase in the half-life of ROS-generating intermediaries.19,24 In cell culture, excessive mitochondrial ROS production activates endothelial inflammatory pathways through activation of both nuclear factor κB and protein kinase Cβ, with subsequent increased expression of endothelial adhesion molecules, increased expression of inflammatory cytokines, and reduced NO bioavailability.17,24 Animal and cell culture data also demonstrate that mitochondrial uncoupling proteins (UCPs) on the inner mitochondrial membrane exert negative feedback on Δψm magnitude and are upregulated in the setting of cellular stressors.31 Overexpression of UCPs suppresses both Δψm magnitude and mitochondrial superoxide, whereas suppression of UCPs increases mitochondrial ROS production.10,17 Overexpression of UCPs in a rat model is protective against free fatty acid–induced endothelial dysfunction and vascular inflammation, whereas decreased UCP-2 expression is associated with increased atherosclerosis in mouse models.16,32 Recent cross-sectional data suggest that a human loss-of-function UCP-2 polymorphism is associated with an adverse cardiovascular risk profile.33 Our data extend these reports by demonstrating the importance of Δψm as a novel, rapid-acting regulator of arteriolar endothelial function in humans with T2DM.

![Figure 4. Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) exposure improved NO bioavailability in arterioles from humans with type 2 diabetes mellitus (T2DM); 61±24 vs 85±29 for diaminofluorescein diacetate (DAF2-DA) fluorescence without and with CCCP, respectively. *P=0.04 by paired t test. Error bars represent ±SE.](http://atvb.ahajournals.org/)

![Figure 5. Effect of MitoTEMPOL on endothelium-dependent vasodilation in human arterioles from patients with type 2 diabetes mellitus (T2DM). MitoTEMPOL (1 mmol/L) for 30 minutes before vasodilator testing significantly improved arteriolar endothelial function (n=4). P<0.001 overall for interaction of acetylcholine (Ach) dose and MitoTEMPOL exposure (A). L-Nω-nitroarginine methyl ester (L-NAME) significantly inhibits the ameliorative effect of MitoTEMPOL on T2DM Ach–induced vasodilation (P<0.001 overall between curves) (B). *P<0.005 between curves at the indicated doses of Ach. Error bars represent ±SE.](http://atvb.ahajournals.org/)
Furthermore, our data suggest that $\Delta \psi_m$ likely alters the endothelial phenotype, at least in part, through modulating mitochondrial ROS production.

We observed reduced mitochondrial mass in arterioles from patients with T2DM relative to nondiabetic subjects. Furthermore, reduced mitochondrial mass was associated with impaired systemic endothelial function, whereas increased mitochondrial superoxide production was associated with both impaired systemic endothelial function and reduced mitochondrial mass. A growing body of work supports key links between cellular mitochondrial morphology and mass with ATP and ROS production in insulin-resistant states.\textsuperscript{10,23} Insulin-resistant offspring of humans with T2DM have reduced mitochondrial density and ATP production capacity,\textsuperscript{23,30} and mononuclear cells from patients with T2DM have smaller, less complex mitochondria.\textsuperscript{20} Interestingly, endothelial NO synthase–dependent NO bioavailability in cell culture and animal models has been shown to be a central regulator of transcription factor PGC-1$\alpha$–dependent mitochondrial biogenesis.\textsuperscript{34} NO-deficient mice develop obesity and insulin resistance in concert with reduced mitochondrial mass.\textsuperscript{34} Furthermore, in the endothelium, suppression of NO bioavailability leads to excessive mitochondrial superoxide production.\textsuperscript{35} Our data extend these findings to the human vasculature by correlating systemic reductions in mitochondrial mass with impairment of NO-dependent endothelial function as well as demonstrating a reduction in mitochondrial mass in the vascular endothelium of patients with T2DM.

Our studies have several limitations. First, the correlations between mitochondrial mass and in vivo measurements of endothelial function are relatively modest. However, the strengths of these correlations are similar to those for traditional cardiovascular risk factors and both FMD and PAT.\textsuperscript{1.8} Because of the study size, we cannot make clear determinations as to whether T2DM or non-T2DM subjects are driving these correlations. Our correlations among glycosylated hemoglobin, fasting glucose, and Ach-induced vasodilation involve a small sample size and need to be repeated in a larger data set to confirm our findings. Second, we cannot infer a causal relationship between reduced mitochondrial mass and endothelial dysfunction on the basis of our data alone. Further in vitro and potentially in vivo work to determine whether manipulations of mitochondrial mass alter endothelial function in humans will be necessary to fully elucidate this potential relationship. We could not withdraw medical therapy from patients with T2DM for $>$24 hours for ethical reasons. Although we cannot exclude medication effects on our arteriolar measurements, the only medication to significantly influence mononuclear cell measures of mitochondrial function was ARB therapy in patients with T2DM. This therapy was associated with a significantly higher mitochondrial mass by NAO fluorescence within the group with T2DM ($241\pm106$ [n=8] versus $98\pm74$ AU [n=27], $P<0.05$). We consider this finding to be hypothesis-generating only. In light of the relative insulin resistance of our control group, it is possible that the differences we observed between our T2DM and non-T2DM groups would be larger if the non-T2DM group had greater insulin sensitivity. We did not collect glycosylated hemoglobin data on these subjects; therefore, we cannot directly determine an association between our vessel findings and near-term glycemic control. Balanced against these limitations are the novelty of the findings and their potential implications for vascular regulation and therapeutic targets in T2DM- and hyperglycemia-associated vascular disease.

Conclusion

Our data demonstrate that mitochondrial hyperpolarization and excessive superoxide production are directly associated with the development and maintenance of vascular endothelial dysfunction in patients with T2DM. Furthermore, reduced mitochondrial mass is significantly associated with impaired NO-dependent endothelial function. Our work suggests mitochondrial dysfunction may play a particularly key role in the development of atherosclerosis and vascular complications in patients with T2DM. Future work to determine and evaluate the efficacy interventions targeted to reduce $\Delta \psi_m$ mitochondrial ROS production, and increase mitochondrial mass in T2DM is warranted.

Acknowledgments

We thank Emily Arthur, Jennifer Multerin, and Dorothee Weihrauch for their help with data acquisition.

Sources of Funding

Dr Widdelansky receives support from K23HL089326, HL081587, the Elsa Shoeneicht Medical Research Fund (Greater Milwaukee Foundation), and a T. Franklin Williams Scholars Award provided by Atlantic Philanthropies, the American Heart Association (Grant-in-Aid 10GRNT13980044), the John A. Hartford Foundation, and the Association of Specialty Physicians. This work was supported by the Clinical Translational Research Institute at the Medical College of Wisconsin and National Institutes of Health (NIH) grant HL081587. Drs Kizhakekuttu, Dharmashankar, and Wang have received support from a Ruth L. Kirschstein NIH T32 training grant (HL007792-15). Dr Guttermar is supported by HL094971 and HL080704. Dr Vita is supported by HL083269, HL083801, HL081587, and HL75759. The contents of this manuscript are solely the responsibility of the authors and do not necessarily represent the official views of the NIH.

Disclosures

None.

References


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Arterioscler Thromb Vasc Biol. published online August 9, 2012;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2012/08/09/ATVBAHA.112.256024

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Tinoy J. Kizhakekuttu, MD,1* Jingli Wang, MD, PhD,1* Kodlipet Dharmashankar,1 Rong Ying,1 David D. Gutterman MD,1,2 Joseph A. Vita, MD3 Michael E. Widlansky, MD, MPH,1,2

1- Department of Medicine, Division of Cardiovascular Medicine, Medical College of Wisconsin
2- Department of Pharmacology, Medical College of Wisconsin
3- Evans Department of Medicine, Boston University School of Medicine
4- Linus Pauling Institute, Oregon State University

*Each author contributed equally to the development of this manuscript

Main Text Word Count: 616 + 2 Tables and 3 Figures

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All data mean±SD
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