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

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### Abstract

**Objective**—Mitochondrial dysfunction plays a key pathophysiological role in type 2 diabetes (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 (FMD%) and digital pulse amplitude tonometry (PAT). Endothelial function in arterioles isolated from gluteal subcutaneous adipose was measured by videomicroscopy. In arterioles and mononuclear cells, we measured inner mitochondrial membrane potential ( $\Delta\psi_m$ ), mitochondrial mass, and mitochondrial superoxide production using fluorophores. Endothelial function was impaired in T2DM versus non-diabetics.  $\Delta\psi_m$  magnitude was larger and mitochondrial mass was lower in arterioles and mononuclear cells in T2DM. Mononuclear mitochondrial mass correlated with FMD% and PAT ( $r=0.38$  and  $0.33$ ,  $P=0.001$  and  $0.02$ , respectively) and mononuclear mitochondrial superoxide production inversely correlated with FMD% ( $\rho=-0.58$ ,  $P=0.03$ ). Endothelial function was impaired in T2DM. Low doses of two different mitochondrial uncoupling agents (CCCP and DNP) that reduce  $\Delta\psi_m$  magnitude and a mitochondrial-targeted antioxidant (MitoTEMPOL) improved endothelial function and reduced mitochondrial superoxide levels.

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

### Keywords

mitochondria; endothelium; diabetes mellitus type 2; nitric oxide; oxidative stress

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Disclosures:

None.

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## Introduction

The worldwide incidence of type 2 diabetes is expected to exceed 350 million persons in 2030, with the incidence rate of type 2 diabetes increasing in all segments of the population worldwide.<sup>1, 2</sup> The majority of morbidity and mortality related to diabetes is secondary to the macrovascular (myocardial infarctions, stroke) and micro-vascular complications (blindness, renal failure) of this disease.<sup>2, 3</sup> The pathogenesis of clinically overt vascular disease in type 2 diabetes (T2DM), while not completely elucidated, appears to begin with the initial development of vascular endothelial dysfunction.<sup>4-8</sup> Endothelial dysfunction encompasses the pro-vasoconstrictive, pro-thrombotic, and pro-inflammatory endothelial phenotype known to precede the development of clinically relevant vascular disease and portend future cardiovascular risk.<sup>9, 10</sup>

The molecular origins of insulin-resistance related vascular endothelial dysfunction include the induction of excessive oxidative stress<sup>7, 11</sup> and the activation pro-inflammatory and pro-vasoconstrictive pathways involving NF- $\kappa$ B and protein kinase C beta (PKC $\beta$ ).<sup>12-14</sup> Hyperglycemia and excess circulating free fatty acids appear to be responsible for the initiation of vascular oxidative stress and inflammation.<sup>15, 16</sup> Interestingly, in cell culture models, activation of these deleterious pathways also appear to require the development of mitochondrial dysfunction.<sup>17, 18</sup> Based on prior cell culture and animal work, insulin-resistance related mitochondrial dysfunction is typified by two key alterations in the mitochondrial phenotype leading to excessive mitochondrial ROS production: inner mitochondrial membrane hyperpolarization<sup>19, 20</sup> and reduced mitochondrial mass and mitochondrial network complexity.<sup>20-22</sup> We recently observed such alterations in mitochondrial function and morphology in peripheral blood mononuclear cells and isolated venous endothelial cells from T2DM patients.<sup>20, 23</sup>

Excessive mitochondrial ROS production is known to cause deleterious vascular cell signaling and subsequent endothelial dysfunction.<sup>24</sup> Despite cell and animal data supporting a potential role for mitochondrial dysfunction in the origins of vascular dysfunction in type 2 diabetes, 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 for T2DM patients would display phenotypical mitochondrial dysfunction with reduced mitochondrial mass and greater mitochondrial inner membrane polarization relative to non-diabetic humans and 3) mitochondrial hyperpolarization and excessive mitochondrial superoxide production would relate mechanistically to arteriolar endothelial dysfunction in T2DM patients.

## Methods

### Subjects

The study protocol and advertisements were approved by the Medical College of Wisconsin's Institutional Research Board. The inclusion/exclusion criteria for non-diabetic subjects and T2DM subjects were previously described.<sup>20</sup> A total of 122 individuals between the ages of 35 and 70 years of age were consecutively enrolled. We previously reported mitochondrial membrane potential, mass, and superoxide production from 59 of these subjects (27 subjects with T2DM, 32 non-T2DM).<sup>20</sup> For T2DM subjects, the additional cohort of 33 subjects had slightly higher systolic blood pressure ( $131\pm 3$  vs.  $125\pm 8$ ,  $P=0.04$ ), heart rate ( $70\pm 2$  vs.  $64\pm 5$ ,  $P=0.046$ ), and fasting triglycerides ( $151\pm 14$  vs.  $128\pm 9$ ,  $P=0.02$ ) but otherwise did not differ with respect to other baseline characteristics in **Table 2**. 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.<sup>25</sup> Non-diabetic control subjects were excluded if they met criteria for metabolic syndrome,<sup>26</sup> had a history of cardiovascular disease by history or documented myocardial infarction,<sup>27</sup> 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 prior to their study visits, and all studies began between 7:30AM and 9:00AM. Subjects on anti-hypertensive medications were asked to hold these medications for 24 hours prior to their study visits. Subjects on oral diabetes 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 (HOMA-IR= [(fasting insulin  $\mu$ IU/mL) $\times$  (fasting glucose mmol/L)]/22.5) and the quantitative insulin-sensitivity check index (QUICKI=1/[log(Insulin) + log(glucose)]).

## Materials

Acetylcholine (Ach), 2,4 dinitrophenol (DNP- reduces mitochondrial membrane potential magnitude,  $\Delta\psi_m$ ), carbonyl cyanide m-chlorophenyl hydrazone (CCCP- also reduces  $\Delta\psi_m$  magnitude), and L- $N^G$ -nitroarginine methyl ester (L-NAME- nitric oxide synthase inhibitor) were purchased from Sigma-Aldrich (St. Louis, MO). 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzamidazol-carboncyanine (JC-1- measures  $\Delta\psi_m$  magnitude), acridine orange 10-nonyl bromide (NAO- measures mitochondrial cardiolipin content as a marker of mitochondrial mass), MitoSox<sup>TM</sup>, diaminofluorescein diacetate (DAF-2DA- measures nitric oxide bioavailability) and tetramethylrhodamine methyl ester (TMRM- measures  $\Delta\psi_m$  magnitude) were purchased from Invitrogen (Carlsbad, CA). MitoTEMPOL was purchased from Enzo Life Sciences International, Inc (Plymouth Meeting, Pennsylvania).

## Measurements of *in vivo* Endothelial Function

**Brachial Artery Reactivity Testing**—We measured flow mediated dilation (FMD%), nitroglycerin mediated dilation(NMD%), flow velocity, and shear stress in the brachial artery as previously described.<sup>28</sup> We recorded average diameter of the entire R-R interval and used these measurements to calculate both FMD% and NMD%. For FMD%, our intra- and inter-observer correlation coefficients are 0.99 and 0.87, respectively. The mean absolute difference measured between observers is  $1.0\pm 0.8\%$ .

**Digital Pulse Amplitude Tonometry**—Digital pulse amplitude was measured using a 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 ratios of baseline pulse amplitudes and post-deflation pulse amplitudes 90-120 seconds post cuff deflation as described previously.<sup>6</sup> Greater detail on the methods for PAT can be found in the on-line supplement.

## Measurement of *in vitro* Endothelial Function

Please see the on-line supplement for full methodological details on the procedure used to obtain human gluteal adipose arterioles and preparation of these vessels for study of endothelium-dependent and independent vasodilation by videomicroscopy. Our success rate for obtaining usable arterioles for study by biopsy was 75%. As each biopsy yielded approximately 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 acetylcholine from  $10^{-10}$  to  $10^{-5}$  M. The eNOS dependence of vasodilation was determined

by concomitant L-NAME exposure (1mM). To determine whether partial depolarization of the mitochondrial inner membrane altered endothelial function, vessels were additionally exposed (intra-luminally) to either CCCP (100 nM), DNP (50 nM) for 30 minutes prior to measurements of acetylcholine-induced vasodilation (flow rate < 5 dynes/cm<sup>2</sup>). To determine whether lowering mitochondrial superoxide levels improved endothelial function, a subset of vessels were intra-luminally exposed to MitoTEMPOL (1 mM) for 30 minutes prior to measuring acetylcholine-induced vasodilation. All vessels were exposed to 0.2 M 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 nM), JC-1 (100 nM), or TMRM (1  $\mu$ M) was allowed to flow through the vascular lumen for 30 min at a very low rate (shear < 5 dynes/cm<sup>2</sup>). Prior to 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) prior to 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 **Supplemental Figures I and II**. NAO and TMRM fluorescence intensities were measured by fluorescent microscopy (Eclipse TE 200, Nikon, Japan) with Ex/Em of 492/ 526nm 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 and 578 nm. All fluorescence intensities in this study were analyzed by using MetaMorph 6.1 (Universal Imaging, West Chester, PA).

**MitoSox<sup>TM</sup> and DAF2-DA Measurements on Arterioles from Subjects with and without T2DM**—Arterioles were incubated in HEPES buffer under normal glucose conditions (5 mM) with either MitoSox<sup>TM</sup> (5  $\mu$ M) or DAF2-DA (5  $\mu$ M) for 30 minutes. MitoSox<sup>TM</sup> fluorescence intensity was measured by fluorescent microscopy (Eclipse TE 200, Nikon, Japan) at Ex/Em 510/572 nm for MitoSox<sup>TM</sup> or 492/526 nm for DAF2-DA. Negative control images from the same vessels were taken before giving fluorescent dye. Reported MitoSox<sup>TM</sup> and DAF2-DA fluorescence intensities are the net of the MitoSox<sup>TM</sup> or DAF2-DA exposed arteriole from each individual minus the individual's matched negative control vessel background fluorescence.

### Systemic Measurements of Mitochondrial Homeostasis

Measurements of mitochondrial superoxide production using MitoSox<sup>TM</sup>, mitochondrial membrane potential ( $\Delta\psi_m$ ) using JC-1, and mitochondrial cardiolipin content as a measure of mitochondrial mass using NAO were performed in mononuclear cells isolated from venous blood of subjects with and without T2DM as previously described.<sup>20</sup> Reproducibility of both JC-1 and NAO measurements in human mononuclear cells using these assays has been previously described.<sup>20</sup> Due to limitations on the amount of cells available from each patient for the studies presented, MitoSox<sup>TM</sup> (n=13), 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 non-diabetics and subjects with type 2 diabetes were compared using unpaired t-tests, Wilcoxon rank sum tests, or chi-squared tests as appropriate. Correlations between mononuclear cell measurements of mitochondrial homeostasis and measures of vascular homeostasis were performed using Spearman's  $\rho$ . For significant univariate correlations, stepwise multivariable linear regressions models were constructed to determine if systemic measures of mitochondrial function predicted systemic measures of endothelial function using the following co-variables: age, sex, systolic blood pressure, LDL cholesterol, HDL cholesterol, smoking status, family history of CAD, and fasting glucose. Dose-response curves within each subject group were compared by repeated measures ANOVA and relevant between group comparisons were made using two-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-values < 0.05 were considered significant.

## Results

### Subject Characteristics

A total of 136 subjects were screened and 14 potential non-diabetic participants were excluded due to screening failures, leaving 122 subjects for study. The baseline characteristics of T2DM and non-diabetic subjects are delineated in **Table 2**. Overall, we found no significant differences in age, sex, smoking status, blood pressure, heart rate, total cholesterol, and HDL cholesterol between the two groups. Patients with T2DM were more likely to be African American and have a family history of CAD. Patients with T2DM also had a significantly larger waist circumference, body mass index, higher fasting insulin and glucose levels, greater insulin resistance as measured by HOMA-IR and QUICKI, and higher serum C-reactive protein levels. LDL cholesterol levels were lower in patients with T2DM likely secondary to concomitant HMG CoA reductase inhibitor therapy. The breakdown of medications taken by the T2DM and non-diabetic subjects is delineated in **Supplemental Table I**.

### Systemic Measurements of Mitochondrial Homeostasis and Their Associations with Systemic Endothelial Function

In a subset of 71 of the 122 subjects (35 non-diabetic, 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 non-diabetic, 49 diabetic). As we previously reported in a smaller set of these subjects,<sup>20</sup> NAO fluorescence intensity was significantly lower in patients with T2DM than control subjects, ( $235 \pm 19$  vs.  $127 \pm 17$  A.U. for non-diabetic vs. subjects with T2DM,  $P < 0.001$ ) and mitochondrial membrane potential was significantly more polarized in T2DM subject relative to non-diabetic subjects ( $13.1 \pm 0.8$  vs.  $17.4 \pm 0.9$  for non-diabetic vs. subjects with T2DM,  $P < 0.001$ ). We have also previously reported excess mitochondrial superoxide production in T2DM subjects relative to non-diabetic subjects in a subset of this population.<sup>20</sup> Interestingly, we now found a strong negative correlation between paired measurements of mononuclear cell NAO fluorescence intensity and MitoSox fluorescence intensity ( $N=13$ ,  $\rho=-0.72$ ,  $p=0.006$ , **Supplemental Figure III**).

Results of systemic measures of endothelial function in both T2DM and non-T2DM subjects are summarized in **Supplemental Table II**. FMD% ( $6.2 \pm 1.6$  vs.  $3.7 \pm 1.7\%$ ,  $P < 0.001$ ) and ln

PAT ( $0.74\pm 0.61$  vs.  $0.48\pm 0.42$ ,  $P=0.02$ ) were significantly lower in T2DM patients. There was a trend toward reduced nitroglycerin-mediated dilation in T2DM (NMD%:  $21.9\pm 7.0$  vs.  $19.5\pm 6.3\%$ ,  $P=0.07$ ; NMDmm:  $0.82\pm 0.26$  vs.  $0.73\pm 0.21$ ,  $p=0.05$ ). We found no differences in baseline peak shear ( $42\pm 15$  vs.  $40\pm 14$  dynes/cm<sup>2</sup>, for non-T2DM and T2DM, respectively,  $P=0.36$ ) or peak hyperemic shear ( $78\pm 28$  vs.  $71\pm 23$  dynes/cm<sup>2</sup> for non-T2DM and T2DM, respectively  $P=0.09$ ) between groups.

We report the significant univariate correlations between systemic measurements of endothelial function and monocyte mitochondrial homeostasis in **Figure 1**. NAO fluorescence intensity positively correlated with FMD%, PAT, and NMD% (**Figures 1a-c**). Mitochondrial superoxide production was inversely associated with FMD% (**Figure 1d**). We found no significant correlations between NAO, JC-1, and MitoSox<sup>TM</sup> fluorescence and resting brachial diameter ( $\rho=-0.17$ ,  $-0.06$ ,  $0.31$ ;  $P=0.15$ ,  $0.59$ , and  $0.27$ , respectively), resting shear ( $\rho=-0.08$ ,  $0.02$ ,  $0.03$ ;  $P=0.52$ ,  $0.84$ , and  $0.91$ ), and peak hyperemic shear ( $\rho=0.05$ ,  $0.01$ ,  $0.02$ ;  $P=0.68$ ,  $0.94$ , and  $0.96$ ). There were no significant correlations between JC-1 and MitoSox<sup>TM</sup> fluorescence and NMD% ( $\rho=-0.10$ ,  $-0.31$ ;  $P=0.35$ ,  $0.31$ ) 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%. In a multivariable analysis for independent predictors of FMD% including MitoSox<sup>TM</sup> fluorescence rather than NAO fluorescence, only MitoSox<sup>TM</sup> 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 T2DM Patients

We measured mitochondrial mass,  $\Delta\psi_m$ , and mitochondrial superoxide production in subcutaneous arterioles from randomly 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 ( $54\pm 24$  vs.  $199\pm 58$  A.U. for T2DM ( $n=10$ ) and non-diabetics ( $n=11$ ), respectively, **Figure 2a**), while  $\Delta\psi_m$  was higher in the endothelium of subjects with T2DM as measured by JC-1 ( $2.26\pm 0.08$  vs.  $1.91\pm 0.06$  for T2DM ( $n=5$ ) and non-diabetics ( $n=9$ ), respectively, **Figure 2b**). Additional arterioles exposed to TMRM confirmed our JC-1 findings ( $224\pm 70$  vs.  $56\pm 21$  A.U. for T2DM ( $n=8$ ) and non-diabetics ( $n=8$ ), respectively, **Figure 2c**). There was a strong trend toward increased mitochondria specific superoxide in arterioles from patients with T2DM ( $43\pm 5$  vs.  $26\pm 7$  A.U. for T2DM ( $n=10$ ) and non-diabetics ( $n=9$ ), respectively  $P=0.05$  vs. non-diabetics, **Figure 2d**). In an additional 5 T2DM and 4 non-T2DM subjects, treatment of arterioles with 30 minutes of 100 nM 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 **Supplemental Figure IV**.

### Effect of Partial Mitochondrial Uncoupling and Mitochondrial Antioxidant Exposure on Human Arteriolar Endothelial Function in T2DM and Non-Diabetic Subjects

Subcutaneous arterioles isolated from patients with T2DM demonstrate impaired endothelium-dependent vasodilation compared to non-diabetic controls ( $P<0.001$ , **Figure 3a**). L-NAME completely inhibits acetylcholine induced endothelium dependent vasodilation in these vessels from both T2DM patients and non-T2DM controls. (**Figure**

**3a**). 30-60 minutes of exposure of T2DM arterioles to 100 nM of the mitochondrial uncoupler CCCP to reduce  $\Delta\psi_m$  magnitude completely reversed endothelial dysfunction in T2DM arterioles ( $P=0.02$ , **Figure 3b**). Co-treatment 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 acetylcholine induced vasodilation of vessels from non-diabetic subjects. There were no significant differences in the acetylcholine response in T2DM vessels exposed to CCCP compared to those exposed to DNP ( $P=0.21$ ). No differences in papaverine responses were seen between T2DM and non-diabetics regardless of CCCP or DNP exposure state. In an additional 4 T2DM subjects, we found NO bioavailability significantly increased in arterioles from T2DM vessels following exposure to 100 nm CCCP ( $P=0.03$ , **Figure 4**). Representative images for these experiments are included in **Supplemental Figure V**.

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 (**Supplemental Table III**).

Correlations between paired acetylcholine 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 acetylcholine ( $10^{-5}$  M). When stratified by T2DM status, these statistical significance of correlations likely due to small numbers but their 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 non-diabetic control subjects. These alterations in mitochondrial function were associated with impaired endothelium-dependent vasodilation to acetylcholine, which in 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 NOS 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 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 ( $\Delta\psi_m$  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 non-invasive measures of endothelial vasomotor function.

Patients with T2DM exhibit both conduit vessel and micro-vascular endothelial dysfunction.<sup>4-8</sup> Endothelial dysfunction in this population predates the development of T2DM in humans as hyperglycemia with and without prevalent insulin resistance can induce systemic endothelial dysfunction.<sup>10</sup> While the pathophysiological mechanisms of endothelial dysfunction in T2DM are yet to be fully elucidated, human cross-sectional studies examining other cell types strongly suggest patients with T2DM have impaired mitochondrial function with concomitant reductions in mitochondrial ATP production capacity and increased mitochondrial ROS production which predate their expression of phenotypical T2DM.<sup>20, 29, 30</sup> Cell culture and animal work suggest exposures to high glucose and free fatty acid levels impair endothelial function through induction of excessive mitochondrial oxidative stress.<sup>10, 16, 17, 24</sup> 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 eNOS dependent endothelial dysfunction mechanistically related to mitochondrial ROS production and  $\Delta\psi_m$  hyperpolarization and 2) alterations in mitochondrial mass and ROS production are systemic and are predictive of systemic *in vivo* measures of endothelial function.

Our data support the concept that  $\Delta\psi_m$  is a central regulator of endothelial function in T2DM.<sup>24</sup>  $\Delta\psi_m$  hyperpolarization, occurring secondary to elevated glucose levels, increases ROS production from the mitochondrial electron transport chain due to reduced electron flux and a subsequent increase in the half-life of ROS generating intermediaries.<sup>19, 24</sup> In cell culture, excessive mitochondrial ROS production activates endothelial inflammatory pathways through activation of both NF- $\kappa$ B and PKC $\beta$  with subsequent increased expression of endothelial adhesion molecules, increased expression of inflammatory cytokines, and reduced NO bioavailability.<sup>17, 24</sup> Animal and cell culture data also demonstrate that mitochondrial uncoupling proteins (UCPs) on the inner mitochondrial membrane exert negative feedback on  $\Delta\psi_m$  magnitude and are up-regulated in the setting of cellular stressors.<sup>31</sup> Overexpression of UCPs suppresses both  $\Delta\psi_m$  magnitude and mitochondrial superoxide while suppression of UCPs increases mitochondrial ROS production.<sup>16, 17</sup> Overexpression of UCPs in an rat model is protective against free fatty acid induced endothelial dysfunction and vascular inflammation, while decreased UCP-2 expression is associated with increased atherosclerosis in mouse models.<sup>16, 32</sup> Recent cross-sectional data suggest a human loss-of-function UCP-2 polymorphism is associated with an adverse cardiovascular risk profile.<sup>33</sup> Our data extend these reports by demonstrating the importance of  $\Delta\psi_m$  as a novel, rapid acting regulator of arteriolar endothelial function in humans with T2DM. Further, our data suggest  $\Delta\psi_m$  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 non-diabetic subjects. Further, reduced mitochondrial mass was associated with impaired systemic endothelial function while 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.<sup>10, 23</sup> Insulin resistant offspring of humans with T2DM have reduced mitochondrial density and ATP production capacity,<sup>22, 30</sup> and mononuclear cells from patients with T2DM have smaller, less complex mitochondria.<sup>20</sup> Interestingly, eNOS 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.<sup>34</sup> NO deficient mice develop obesity and insulin resistance in concert with reduced mitochondrial mass.<sup>34</sup> Further, in the endothelium, suppression of NO bioavailability leads to excessive mitochondrial superoxide production.<sup>35</sup> 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.<sup>6, 36</sup> Due to the study size, we cannot make clear determinations as to whether T2DM or non-T2DM subjects are driving these correlations. Our correlations between glycosylated hemoglobin, fasting glucose, and acetylcholine induced vasodilation involve a small sample size and need to be repeated in a larger dataset to confirm our findings. Second, we cannot infer a causal relationship between reduced mitochondrial mass and endothelial dysfunction on the basis 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 patients for greater than 24 hours for ethical reasons. While 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 T2DM patients. This therapy was associated with a significantly higher mitochondrial mass by NAO fluorescence within the group with T2DM [241±106 (n=8) vs. 98±74 A.U. (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. Further, 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 to evaluate the efficacy interventions targeted to reduce  $\Delta\psi_m$ , mitochondrial ROS production, and/or increase mitochondrial mass in T2DM is warranted.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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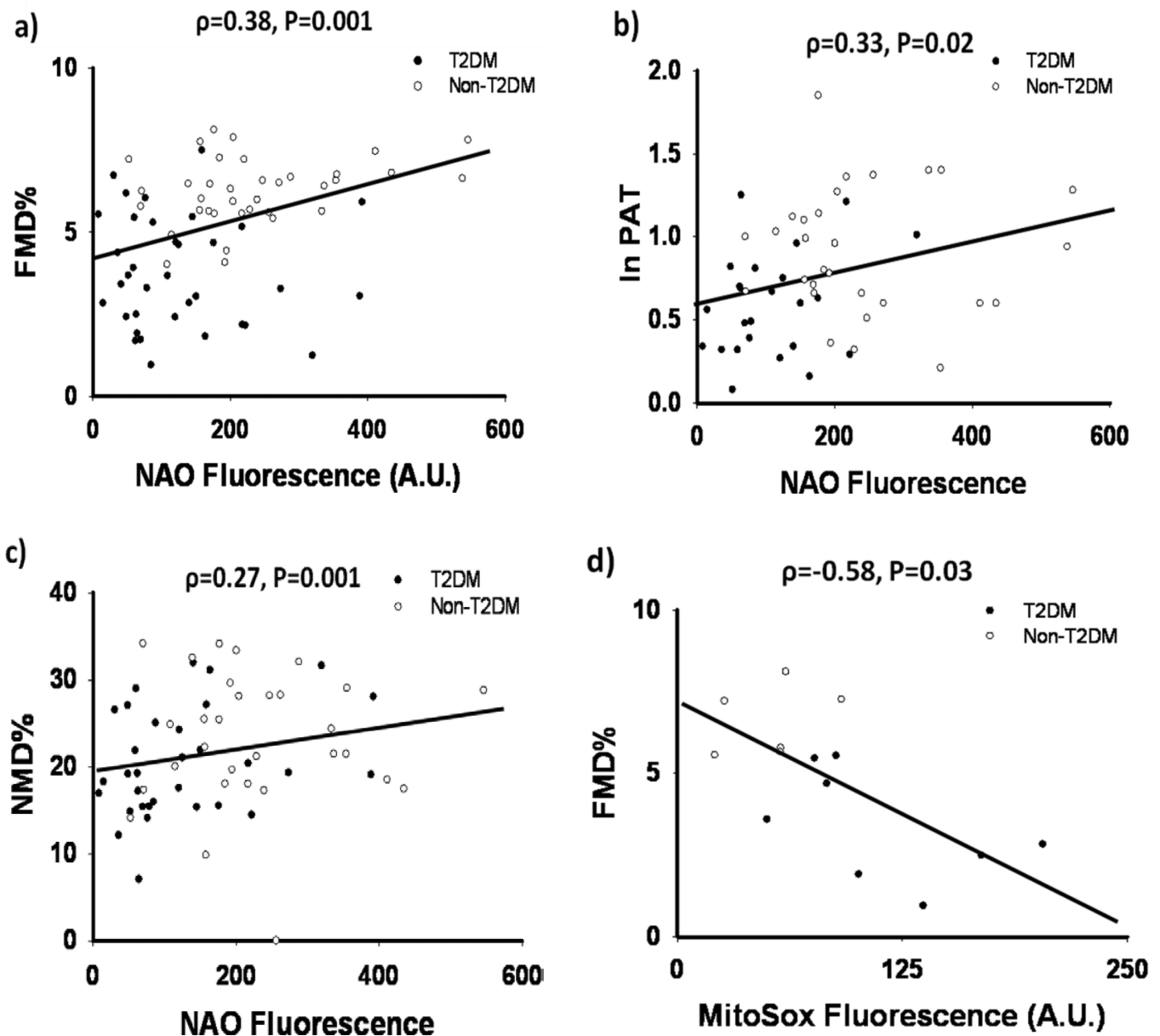
received supported from a Ruth L. Kirschstein NIH T32 training grant (HL007792-15). Dr. Gutterman is supported by HL094971 and HL080704. Dr. Vita is supported by HL083269, HL083801, HL081587, and HL75795. The contents of this manuscript are solely the responsibility of the authors and do not necessarily represent the official views of the NIH.

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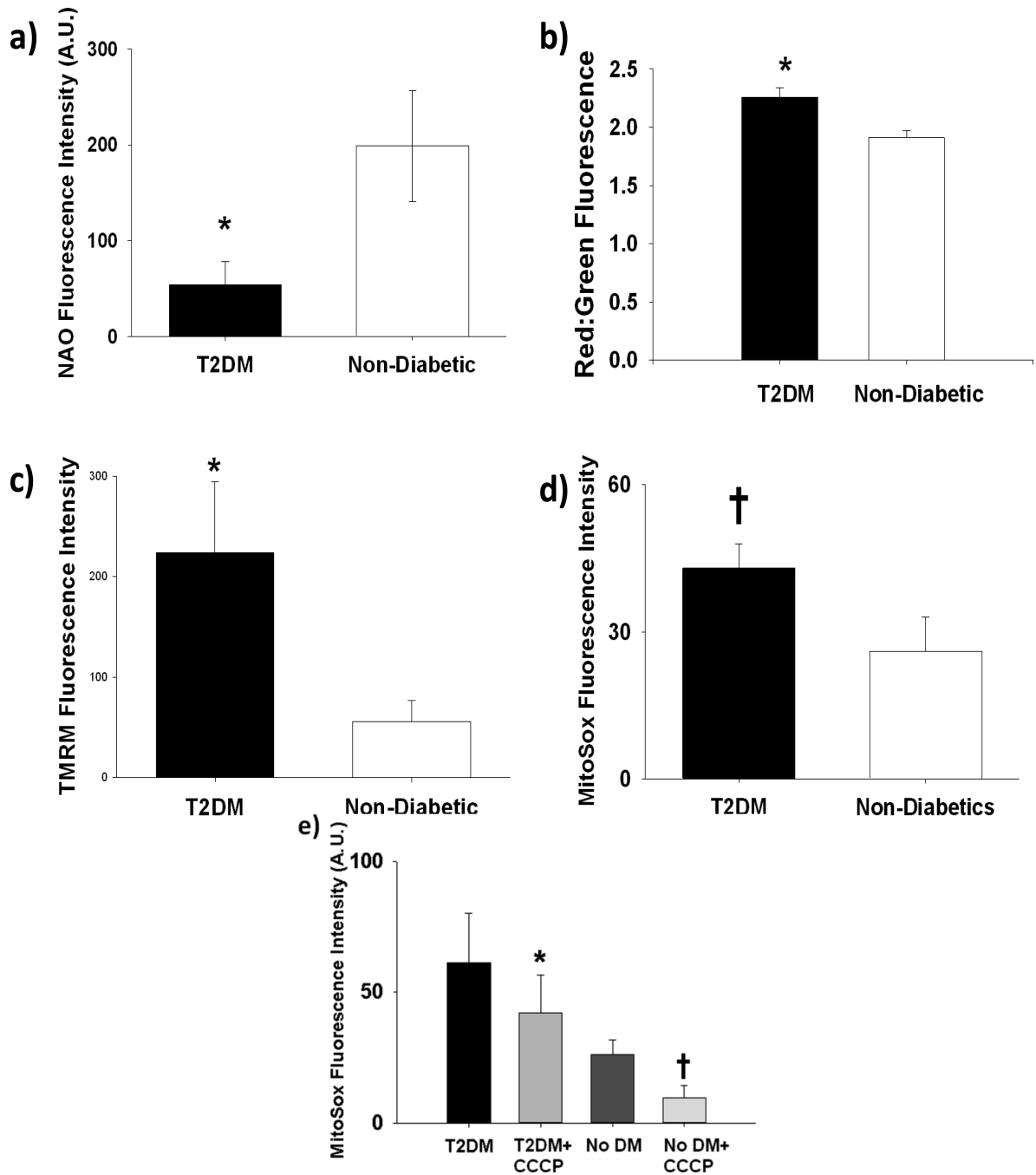
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**Figure 1. Correlations Between Systemic Measures of Endothelial Function and Mitochondrial Homeostasis**

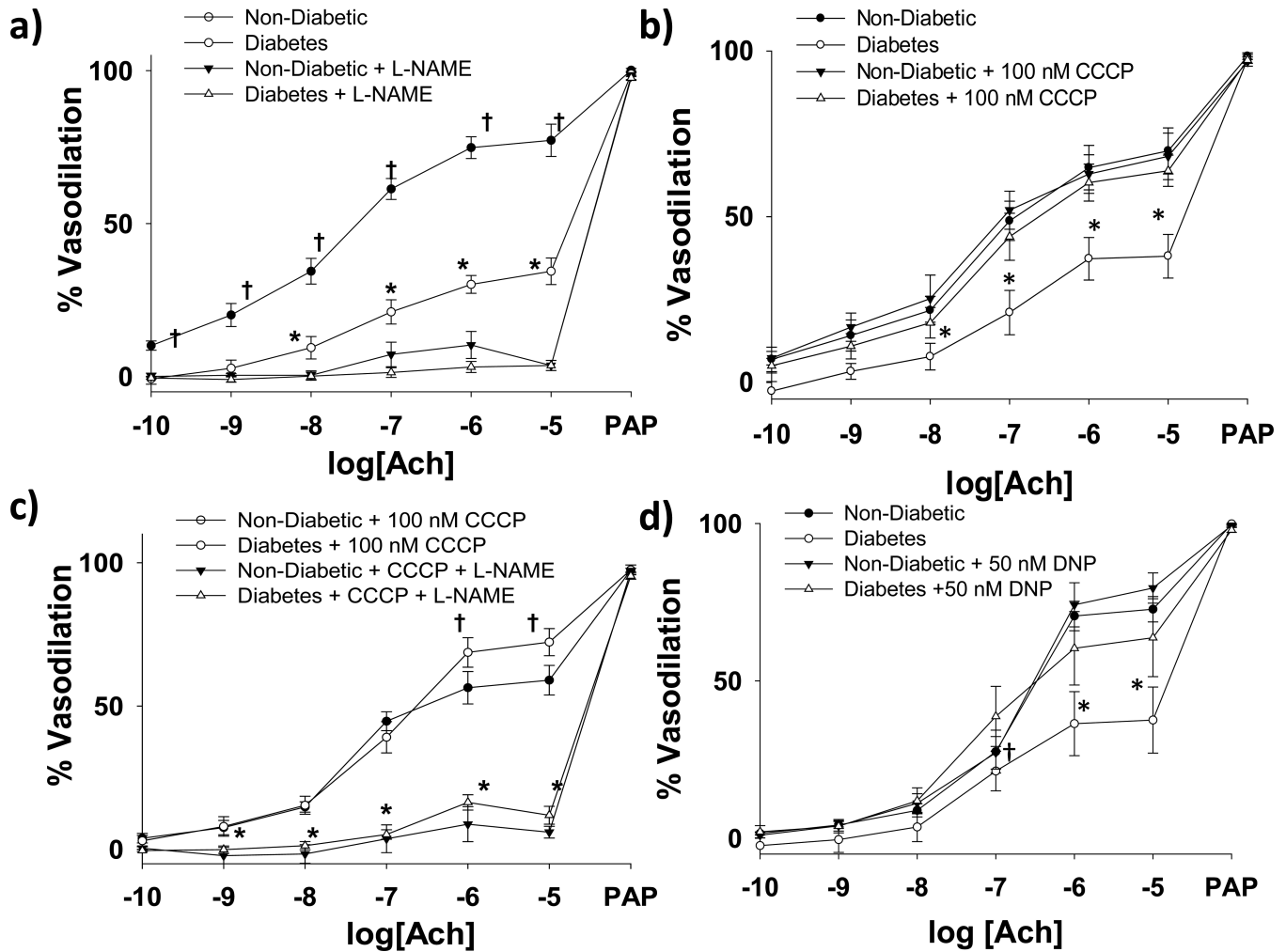
Mitochondrial mass in human monocytes measured by NAO fluorescence intensity was positively correlated with brachial artery flow-mediated dilation ( $\rho=0.38, p=0.001, N=71$ ) (a), digital PAT ( $\rho=0.33, p=0.02, N=53$ ), (b) brachial artery nitroglycerin-mediated dilated, ( $\rho=0.27, p=0.001, N=62$ ) (c). Mitochondrial superoxide production in human monocytes showed a strong negative correlation with FMD%, ( $\rho=-0.58, p=0.03, N=14$ )(d).



**Figure 2. Comparisons of Measurements of Mitochondrial Homeostasis in the Arterioles of Humans with and without T2DM**

The arteriolar endothelium of patients with T2DM had significantly reduced mitochondria mass as reflected by reduced NAO fluorescence ( $54 \pm 24$  vs.  $199 \pm 58$  A.U., N=10 and 11 for T2DM and non-T2DM, respectively, \*P=0.04) (a), increased  $\Delta\psi_m$  by both JC-1 ( $2.26 \pm 0.08$  vs.  $1.91 \pm 0.06$ , N=5 and 9 for T2DM and non-T2DM, respectively, \*P=0.01) (b) and TMRM ( $224 \pm 70$  vs.  $56 \pm 21$  A.U., N=8 and 8 for T2DM and non-T2DM, respectively, \*P=0.04) (c). A strong trend toward an increase in mitochondrial superoxide was also seen in T2DM arterioles versus control subjects ( $43 \pm 5$  vs.  $26 \pm 7$  A.U., N=10 and 9 for T2DM and non-T2DM, respectively, \*P=0.05) (d). Treatment with CCCP (100nM) significantly

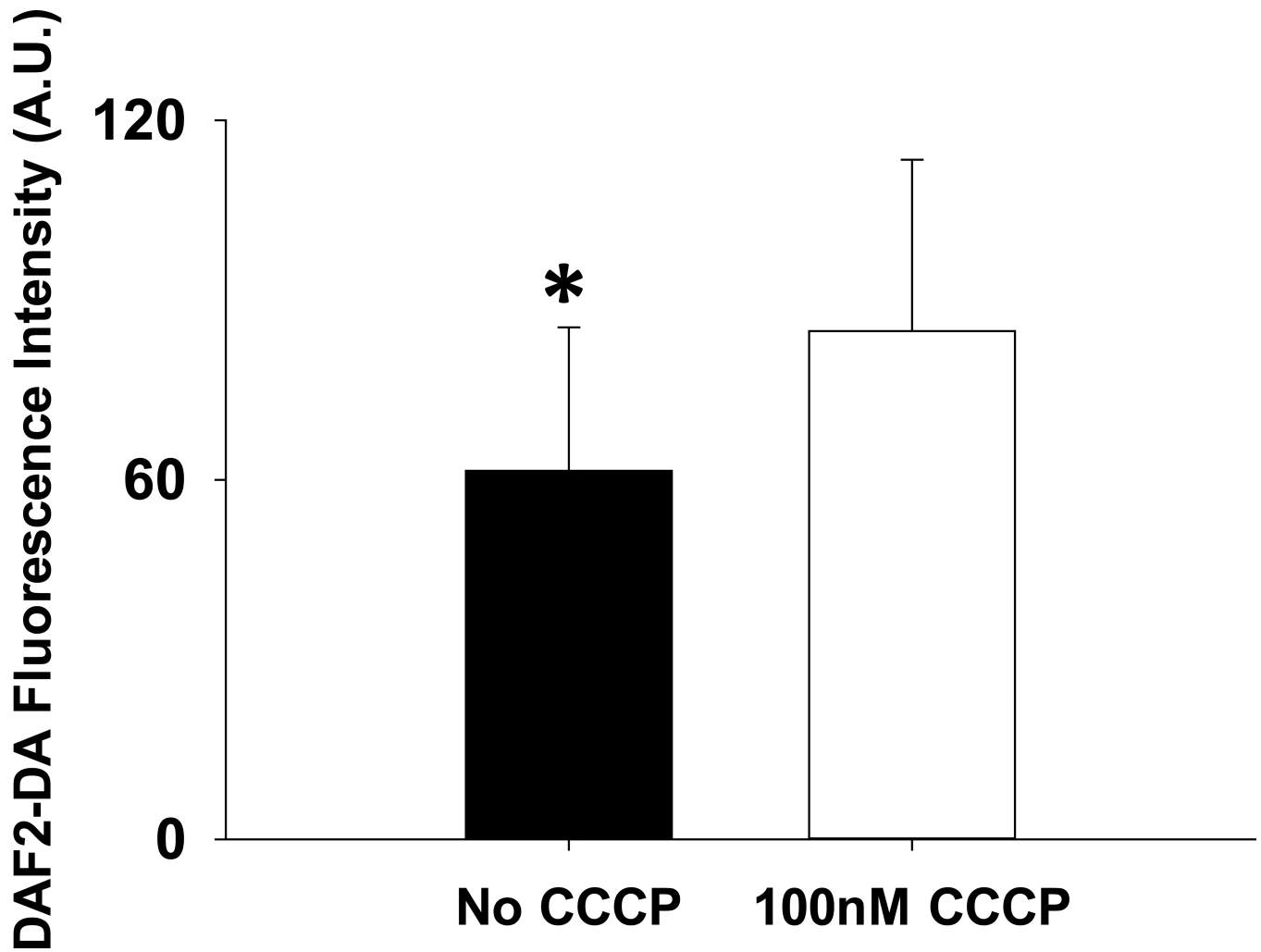
reduced mitochondrial superoxide production in both T2DM subjects ( $61 \pm 42$  and  $42 \pm 31$  for T2DM and T2DM+CCCP, respectively, N=5) and non-T2DM subjects ( $37 \pm 27$  and  $22 \pm 10$  for non-DM and non-DM +CCCP, respectively N=4) (P=0.002 overall by mixed ANOVA). \*-P<0.05 vs. non-diabetics, †-P=0.05 vs. non-diabetics (e). Error bars represent  $\pm$  SE.



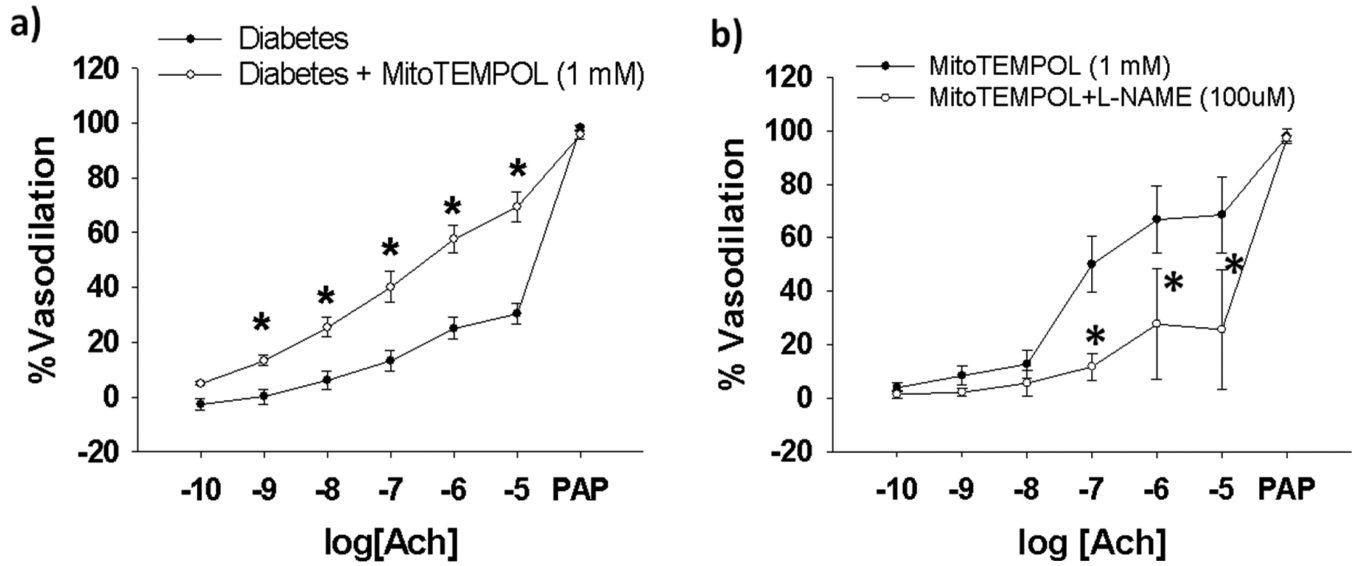
**Figure 3. eNOS-related Endothelium Dependent Vasodilation in Arterioles from Patients with T2DM**

Impairment in endothelial function in T2DM arterioles is eNOS dependent (N=8 for T2DM and N=8 for controls.  $P < 0.001$  overall,  $P < 0.001$  between curves with and without L-NAME for both T2DM and non-T2DM, respectively.  $*P < 0.05$  for T2DM vs. all other exposure at the indicated Ach concentrations, †-  $P < 0.05$  vs. non-T2DM + L-NAME at the indicated Ach dose) (a). Exposure to 100 nM CCCP for 30 minutes reverses T2DM related endothelial dysfunction (N=6 controls, N=6 T2DM.  $P < 0.001$  overall and  $P = 0.002$  and  $0.71$  between curves with and without CCCP for T2DM and non-T2DM subjects, respectively.  $*P < 0.05$  vs. all other exposures at the indicated Ach dose) (b). Improved endothelial dysfunction with CCCP is related to improved eNOS activity (N=5 controls, N=6 T2DM.  $P < 0.001$  between curves with and without L-NAME for both T2DM and non-T2DM subjects,  $*P < 0.05$  for both non-diabetic + CCCP + L-NAME and diabetes + CCCP + L-NAME vs. both non-diabetic + CCCP and T2DM + CCCP. †-  $P < 0.05$  for T2DM + CCCP vs. non-diabetic + CCCP) (c) Exposure to 50 nM DNP also improved endothelial function in T2DM arterioles (N=7 controls, N=6 T2DM.  $P = 0.01$  overall,  $P = 0.01$  and  $P = 0.44$  between curves with and without DNP for T2DM and non-T2DM subjects, respectively.  $*P < 0.05$  vs. all other exposures at the indicated Ach dose. †-  $P < 0.05$  vs. T2DM alone only) (d). Ach-Acetylcholine. PAP- Papaverine. Error bars represent  $\pm$  SE.





**Figure 4. CCCP Exposure Improved NO Bioavailability in Arterioles from Humans with T2DM**  $61 \pm 24$  vs.  $85 \pm 29$  for DAF2-DA fluorescence without and with CCCP, respectively. \* -  $P=0.04$  by paired t-test. Error bars represent  $\pm$  SE.



**Figure 5. Effect of MitoTEMPOL on Endothelium-Dependent Vasodilation in Human Arterioles from Patients with Type 2 Diabetes**

MitoTEMPOL (1 mM) for 30 minutes prior to vasodilator testing significantly improved arteriolar endothelial function (N=4,  $P < 0.001$  overall for interaction of acetylcholine dose and MitoTEMPOL exposure (a)). L-NAME significantly inhibits the ameliorative effect of MitoTEMPOL on T2DM acetylcholine induced vasodilation ( $P < 0.001$  overall between curves) (b). \* -  $P < 0.005$  between curves at the indicated doses of acetylcholine). Ach-acetylcholine. Error bars represent  $\pm$  SE.

**Table 1**

## List of Abbreviations

<b><math>\Delta \Psi_m</math></b>	mitochondrial inner membrane potential
<b>Ach</b>	acetylcholine
<b>CCCP</b>	carbonyl cyanide m-chlorophenyl hydrazone
<b>DAF2-DA</b>	diaminofluorescein diacetate
<b>DNP</b>	2,4-dinitrophenol
<b>eNOS</b>	Endothelial nitric oxide synthase
<b>FMD%</b>	Percent flow mediated dilation
<b>HOMA-IR</b>	homeostasis model assessment-insulin resistance
<b>JC-1</b>	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzamidazol-carboncyanine
<b>L-NAME</b>	L-N <sup>G</sup> -nitroarginine methyl ester
<b>NAO</b>	acridine orange 10-nonyl bromide
<b>NMD%</b>	percent nitroglycerin mediated dilation
<b>PAT</b>	peripheral arterial tonometry
<b>PEG-SOD</b>	pegylated superoxide dismutase
<b>PKC<math>\beta</math></b>	protein kinase C beta
<b>QUICKI</b>	quantitative insulin-sensitivity check index
<b>ROS</b>	reactive oxygen species
<b>T2DM</b>	type 2 diabetes
<b>TMRM</b>	tetramethylrhodamine methyl ester
<b>UCP</b>	mitochondrial uncoupling protein

**Table 2**

## Subject Characteristics

	Type 2 Diabetes (N=60 <sup>*</sup> )	No Diabetes (N=62 <sup>†</sup> )	P-Value
Age	52±9	49±8	0.08
Sex (% Female)	67%	65%	0.80
Race (% African American)	30%	11%	0.009
Body Mass Index (kg/m <sup>2</sup> )	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 (mmHg)	128±29	125±19	0.46
Diastolic Blood Pressure (mmHg)	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 (uU/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

\* N=59 for waist circumference, 59 for glucose, 58 for insulin, 56 for hsCRP, and 23 for glycosylated hemoglobin

† N=60 for glucose, 60 for waist circumference, 59 for insulin, 59 for hsCRP, and 18 for glycosylated hemoglobin.