Supplemental Material

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

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, MD[3] 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

Running Title: Diabetes, Mitochondria, and Endothelial Function

Supplemental Methods:

Digital Pulse Amplitude Tonometry

For each study, the PAT device pressure was set to the lower of 70 mmHg or 10 mmHg below diastolic pressure. Baseline pulse amplitude was measured in both index fingers for 2 minutes. A blood pressure cuff placed on one forearm (the "test" arm) was subsequently inflated to 200 mmHg or 50 mmHg above systolic pressure (whichever was higher) and held at this pressure for 5 minutes. The cuff pressure was subsequently released. Pulse amplitude was measured continuously in the index fingers of the test and control arms throughout the entire study and reported in 30 second intervals for 4 minutes following cuff release.

Human Arteriolar Acquisition and Preparation for Analysis of Endothelial Function

Human subcutaneous arterioles from non-diabetic and T2DM subjects were obtained by gluteal fat pad biopsy under local anesthesia (1% lidocaine) using sterile technique. Following sterilization and the use of local anesthesia, a small (~ 1 cm) incision was made into the gluteal fat pad approximately 2-3 cm medial and cephalad of the greater trochanter. Approximately 2 mL of fat tissue was removed by sharp dissection and incision closed with an absorbable suture and steristrips. The fat sample was transferred immediately into cold HEPES buffer (4ºC) and taken for immediate analysis.

Arterioles were isolated and cleaned of fat and connective tissue. Vessel were subsequently transferred to an organ bath and cannulated with tapered glass micropipettes filled with Krebs buffer consisting of (in mM) 123 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 0.026 EDTA, 1.2 $MgSO_4$, 20 $NaHCO_3$, 1.2 KH_2PO_4, and 5 mM of glucose. The bath was subsequently transferred to the stage of an inverted microscope attached to a video camera and monitor with video calipers. The bath was continuously superfused with Krebs buffer bubbled with a gas

mixture of 21% O_2, 5% CO_2, and 74% N_2. Arterioles were allowed to equilibrate to an intra-luminal pressure of 20 mmHg supplied by a reservoir system for 30 minutes. The intra-luminal pressure was subsequently increased to 60 mmHg for 30 minutes. Following equilibration, vessels were pre-constricted to 50% of maximal diameter with endothelin-1.

Supplemental Figure Legends:

Supplemental Figure I: Endothelial Specificity of JC-1 and NAO Fluorescence in Human Arterioles Using our protocol delineated in the methods section, we imaged 3 human arterioles by confocal microscopy focused on the endothelial cell layer following intra-luminal exposure to JC-1. Both red **(a)** and green **(b)** fluorescence intensity was significantly higher in intact arterioles exposed to JC-1 compared to vessels exposed to buffer alone or denuded of endothelium by air bolus following JC-1 exposure. (*-P=0.01 overall, P <0.05 vs. buffer alone and denuded vessel for both red and green JC-1 fluorescence). Similarly, we imaged 3 human arterioles following intra-luminal exposure to NAO **(c)**. NAO fluorescence intensity was significantly greater in intact arterioles compared to those denuded of endothelium following NAO exposure (*-P=0.02).

Supplemental Figure II: Sample images of from arteriolar studies using NAO **(a-c)** and JC-1 **(d-f)** demonstrating endothelial specificity of the protocols for these fluorophores.

Supplemental Figure III: MitoSox™ negatively correlated with NAO fluorescence intensity in human mononuclear cells isolated from peripheral venous blood (N=14).

Supplemental Figure IV: Representative MitoSox™ fluorescence images from arterioles from a single T2DM subject without CCCP exposure, **(a)**, following 30 minutes of CCCP (100 nM) exposure **(b)**, and a negative control vessel without exposure to DAF2-DA or CCCP **(c)**.

Similar without CCCP **(d)**, with CCCP (100 nM) **(e)**, and negative control images are shown for a non-T2DM subject.

Supplemental Figure V: Representative DAF2-DA (5 µM) fluorescence images from arterioles from a single T2DM subject without CCCP exposure, **(a)**, following 30 minutes of CCCP (100 nM) exposure **(b)**, and a negative control vessel without exposure to DAF2-DA or CCCP **(c)**.

Supplemental Table I – Percentage of Subjects Taking Medications from Listed Classes

Medication	Type 2 Diabetes	Non-Diabetics
Insulin	11.7	0
Metformin	78.3	0
Sulfonylurea	35.0	0
Thiazoladinedione	6.7	0
Dipeptidyl peptidase-4 inhibitors	1.7	0
Incretin Mimetics	1.7	0
Beta-Blockers	20.0	0
ACE Inhibitors	30.0	0
Angiotensin-2 Receptor blockers	18.3	1.6
Calcium Channel Blockers	10.0	0
Diuretic	26.7	0
Hydralazine	3.3	0
Aspirin	33.3	3.2
HMG-CoA Reductase Inhibitors	38.3	0
NSAIDs	6.7	6.5
Multivitamins	30.0	22.6
Hormone Replacement Therapy	3.3%	6.5

Supplemental Table II- *In Vivo* Measurements of Vascular Endothelial Function

	Type 2 Diabetes (N=60)	No Diabetes (N=62)	P-value
Resting Brachial Diameter (cm)	3.9±0.7	3.7±0.7	0.21
Flow Mediated Dilation (%)	3.7±1.7	6.2±1.6	<0.001
Peak Resting Shear (dynes/cm^2)	39.7±14.0	42.1±15.1	0.36
Peak Hyperemic Shear (dynes/cm^2)	70.7±28.3	78.7±28.2	0.09
Nitroglycerin Mediated Dilation (%)*	19.5±6.3	21.9±7.0	0.07
Nitroglycerin Mediated Dilation (mm)	0.73±21	0.82±0.26	0.05
ln PAT (90-120 sec)†	0.48±0.42	0.74±0.61	0.02

*-N=53 for T2DM, N=51 for non-diabetics

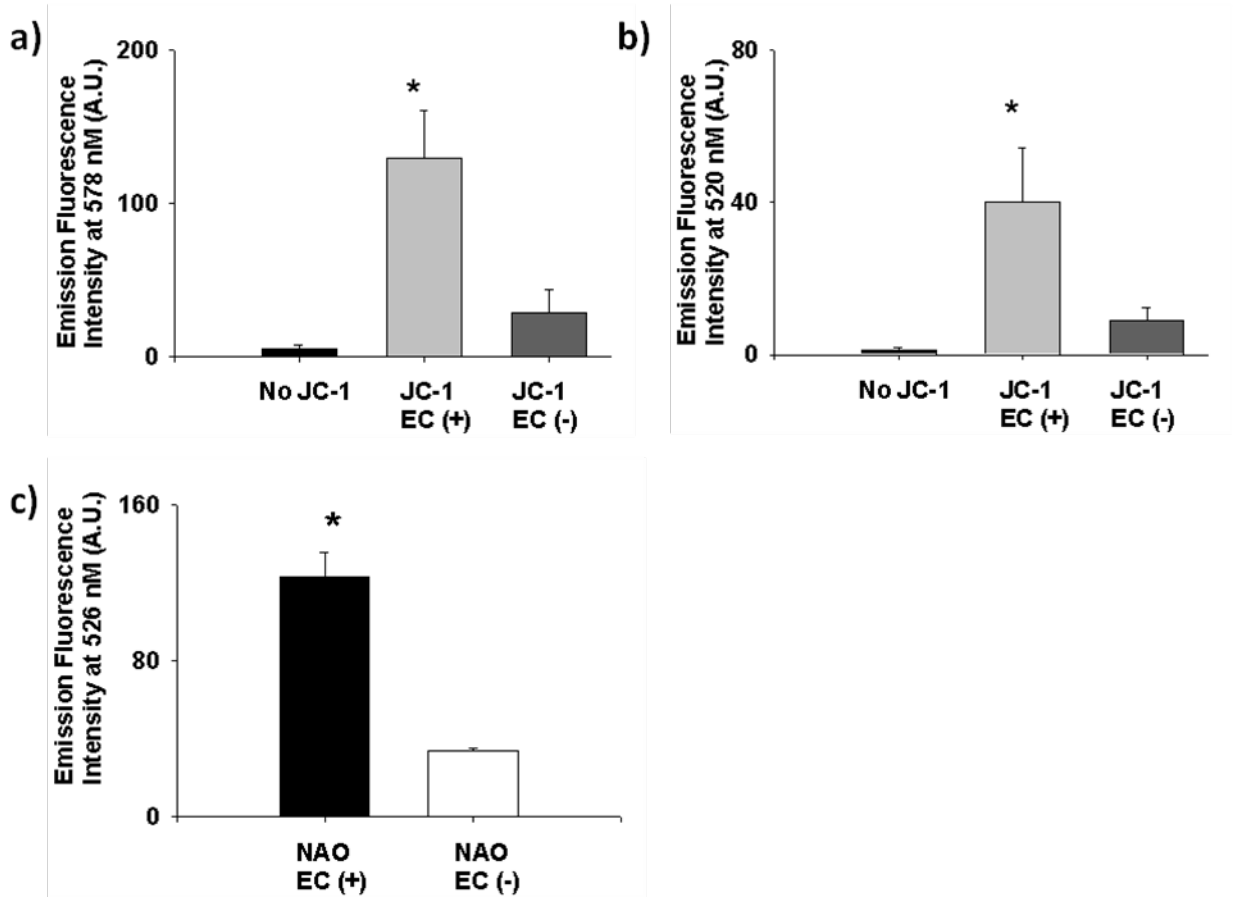
†-N=43 with T2DM, N=47 for non-diabetics

Supplemental Table III- Resting Diameters of Human Arterioles Pre and Post Exposure to Pharmacological Agents

	Resting Diameter (nm) Prior to Exposure	Resting Diameter Post Exposure	P-value
CCCP (n=12)	123±74	121±71	0.94
DNP (n=13)	101±46	93±48	0.67
MitoTEMPOL (n=4)	64±27	61±27	0.84

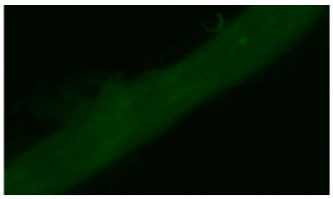
All data mean±SD

Supplemental Figure I

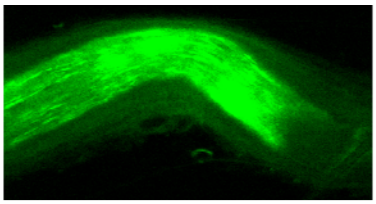


Supplemental Figure II

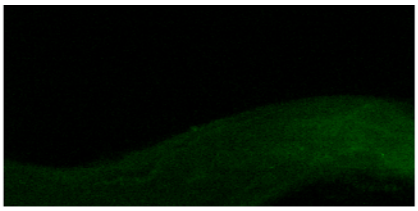
a) Buffer Only



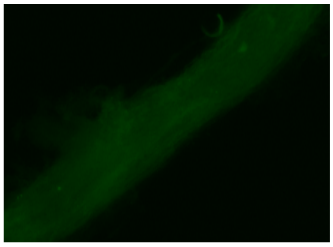
b) NAO Endo(+)



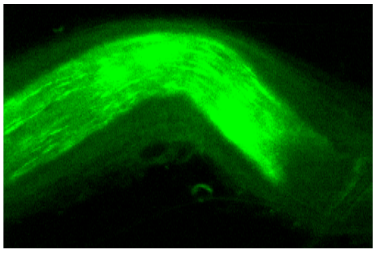
c) NAO Endo (-)



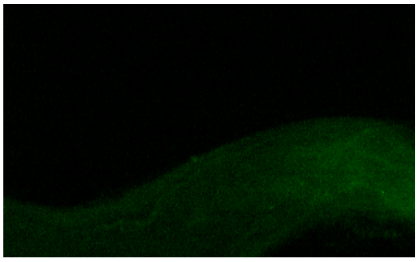
d) Buffer Only



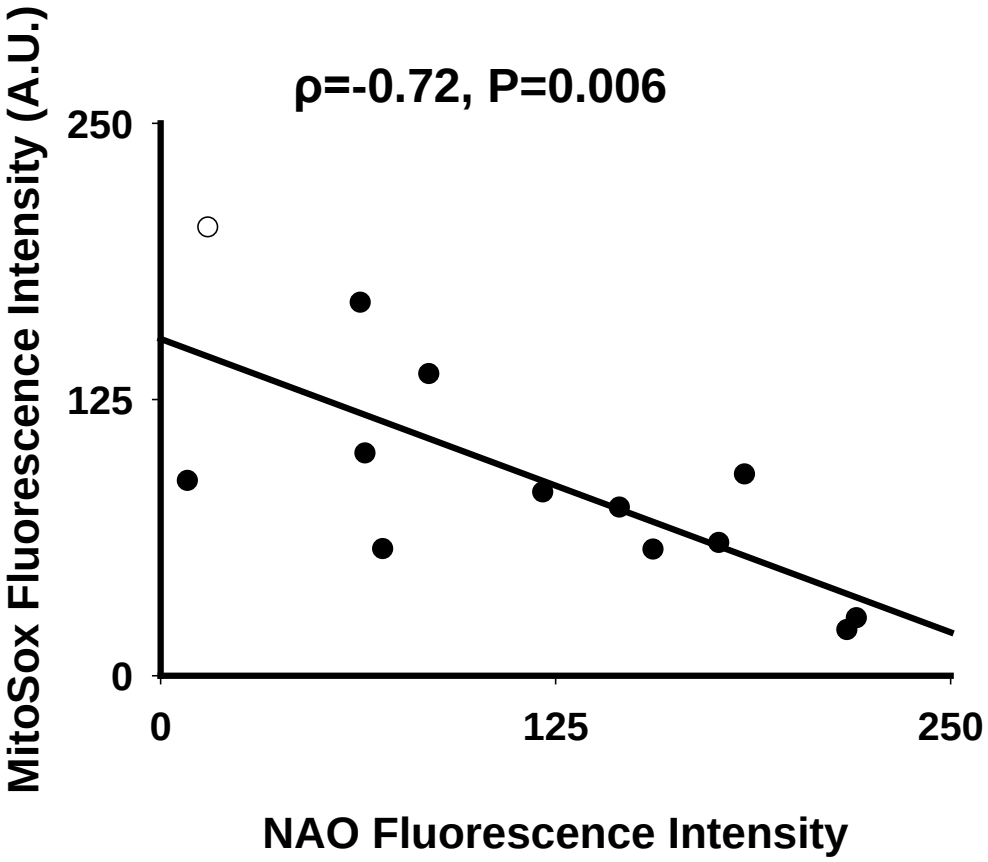
e) JC-1 Endo(+)



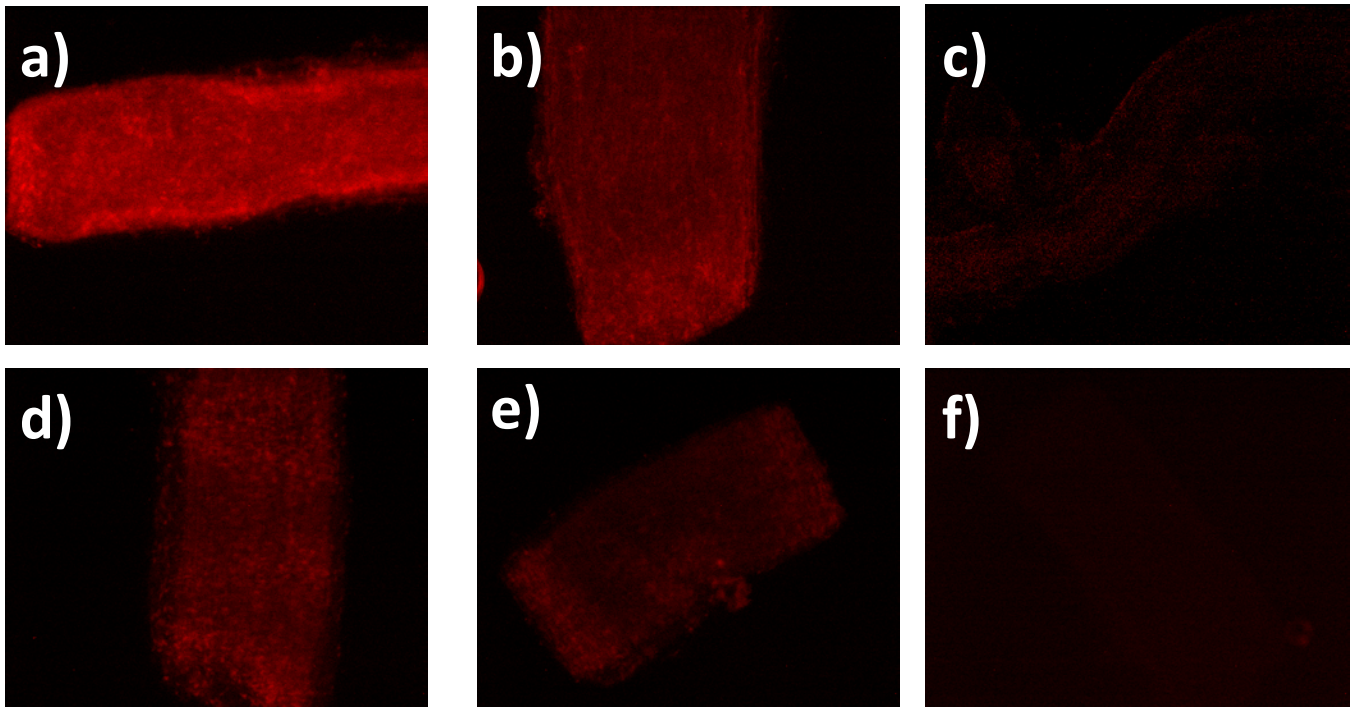
f) JC-1 Endo (-)



Supplemental Figure III



Supplemental Figure IV



Supplemental Figure V