EXTENDED EXPERIMENTAL PROCEDURES

Cell culture, MICU1 and MCU plasmid Constructs, Stable shRNA and Protein Expression

HeLa and COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C, 5% CO_2. EA.hy926 and human ECs were grown in Dulbecco's modified Eagle's medium (DMEM) high glucose with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in the presence of 0.5% endothelial cell growth supplement (ECGS) at 37°C, 5% CO_2. Cytochrome *c* - GFP was obtained from Douglas Green, St Jude Children's Research Hospital, Memphis, TN, USA. COX8A-mRFP, MICU1-YFP, MICU1-ΔK-YFP, HA (MICU1, Δ1, Δ2, Δ3, Δ4 and Δ5), GFP (MCU, Δ1, Δ2 and Δ3) Myc-Flag/DDK (MICU1, ΔEF1, ΔEF2, ΔEF1ΔEF2 and MICU1-ΔK) plasmid constructs were generated by Origene technologies. MICU1 and MCU knockdown cells were grown in DMEM & EC modified media with 2 μg/mL puromycin to create stable knockdown cells (Mallilankaraman et al., 2012a; Mallilankaraman et al., 2012b). All truncation and rescue mutants were grown in DMEM & EC modified media under G418 selective pressure (500 μg/mL).

Isolation of Endothelial Cells From CVD And Control Subjects

Primary ECs were isolated from arteries and veins obtained from individuals undergoing surgical intervention for CAD/PAD at the Surgical Unit of Temple University Hospital after due ethical clearance and informed consent. The tissues were collected in standard EC transport medium and transported to the laboratory through cold chain and processed immediately. The tissues were initially washed once with PBS containing 100 U/ml penicillin, and 100 μg/ml streptomycin, cleaned to remove the fibrous portion with sterile forceps. Using a sterile blade the veins were cut vertically and carefully flipped over. The tissues were cut into small squares of approximately 0.5 cm x 0.5 cm and placed in a 100 mm dish with medium just bathing the tissue pieces. The plates were incubated at 37°C, 5% CO_2. After a week, the tissue pieces were removed leaving the individual colonies to develop into a confluent monolayer. Endothelial cell phenotypes were characterized as previously described (Milovanova et al., 2008).

Dynamic Protein Flux Assay

HeLa cells were co-transfected with cyto *c*-GFP and COX8A-mRFP (mitochondrial targeting sequence; MTS), MICU1-YFP and COX8-mRFP or MCU-GFP and COX8-mRFP plasmid constructs. 48 hours post transfection, cells were permeabilized with digitonin (0.002% wt/vol) for seven minutes. Permeabilized cells were washed with digitonin free intracellular-like medium (ICM buffer, in mM: 120 KCl, 10 NaCl, 1 KH_2PO_4, 20 HEPES-Tris, pH 7.2,) supplemented with 2 mM succinate. Coverslips were mounted

in an open perfusion microincubator (PDMI-2; Harvard Apparatus) at 37°C. After 100 seconds of baseline recording, permeabilized cells were exposed to either mastoparan (20 μg/ml) or alamethicin (20 μg/ml) at the indicated time points. Confocal images were recorded every 10 s (510 Meta; Carl Zeiss, Inc.) at 488- and 561-nm excitation using a 40x oil objective. After completion of the time series, protein distribution maps were generated for before and after the mitochondrial membrane permeabilizing agents. Images were analyzed and flux rate was calculated using ZEN 2010 software and Sigma Plot 11.0.

Detection of Mitochondrial Protein Release by Western Blotting

HeLa cells permeabilized with digitonin (0.002% wt/vol) containing ICM buffer were supplemented with 2 mM succinate for seven minutes. After permeabilization, cells were challenged with mastoparan (20 μg/ml) or alamethicin (20 μg/ml) for 5 min. Supernatant (cytosolic fraction) fractions were separated from the mitochondria containing membranes by centrifugation at 15,000 x rpm for 10 min. Supernatant and membrane fraction proteins were resolved using SDS-PAGE and probed using anti-cytochrome *c* (BD Biosciences), anti-HSP60 (abcam), anti-Flag (Sigma-Aldrich) and anti-GFP antibodies (Axxora).

Fluorescence Recovery After Photobleaching (FRAP) Analysis

HeLa cells transfected with either MCU-GFP or MICU1-YFP were grown on glass coverslips for 48 hours before FRAP experiments. After baseline images were captured (one image/second), a small region (approximately 5% cytoplasmic area) was bleached with 10 iterations of 488 nm at 100% laser power using a Zeiss 510 confocal microscope. Recordings of fluorescence recovery were captured for the following 45 seconds at 488 nm excitation. Recovery in the bleached region was the sum of all diffusing molecules and normalized intensity was calculated as a percentage of the difference between the initial and bleached fluorescent arbitrary unit value. Images were analyzed using Zen 2010.

Immunoprecipitation and Western Blotting

Cell extracts were prepared from either stably or transiently transfected COS-7 cells using RIPA buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.25% deoxycholic acid, 1 mM EDTA, 1% NP-40, protease inhibitor cocktail (Complete: Roche) and 1 mM PMSF). GFP-tagged MCU and its truncations, HA-tagged MICU1 and its truncations, Flag (DDK)-tagged MICU1 and its mutants were immunoprecipitated using anti-GFP (Axxora), anti-HA (Thermo SCIENTIFIC) or anti-DDK (ORIGENE) antibodies. Proteins were resolved using SDS-PAGE and analyzed using anti-GFP, anti-HA, anti-Flag (Sigma-Aldrich) or anti-MICU1 (Sigma-Aldrich) antibodies in the Western blots. CVD

and control ECs for WB were processed as above mentioned and probed with anti-MICU1 (Sigma-Aldrich) and anti cyto *c* antibodies.

Yeast Two-Hybrid Analysis

Full-length MCU, MCU-Δ1, Δ2 and Δ3 cDNA constructs were sub-cloned into the GAL4 DNA binding domain of pGBKT7 (Clontech) so as to serve as "bait." The "prey" was full-length MICU1 cDNA cloned next to the GAL4 activation domain of pGADT7 (Clontech). The bait and prey were transformed into Y2H Gold cells together. Functional interactions were tested by growth of the transformed yeast cells initially plated on medium stringency media containing SD/-Leu/-Trp and then plated onto SD/–Ade/–His/–Leu/–Trp/X-α-Gal/AbA plates, which activate the expression of repression proteins. The empty pGBKT7 vector served as a negative control while the control vectors supplied with the matchmaker Gold Yeast Two-Hybrid System served as a positive control.

Measurement of $[Ca^{2+}]_c$ And $[Ca^{2+}]_m$ in Intact Cell System

HeLa cells grown on 25-mm glass coverslips were loaded with 2 μM rhod-2/AM (50 min) and 5 μM Fluo-4/AM (30 min) in extracellular medium as previously described (Hawkins et al., 2010). Coverslips were mounted in an open perfusion microincubator (PDMI-2; Harvard Apparatus) at 37°C and imaged. After 1 min of baseline recording, histamine (100 μM) was added and confocal images were recorded every 3s (510 Meta; Carl Zeiss, Inc.) at 488- and 561-nm excitation using a 40x oil objective. Images were analyzed and quantitated using ZEN 2010 software.

FRET acceptor photobleaching assay

FRET acceptor photobleaching experiments were performed using HeLa cells co-transfected with MCU-GFP/MICU1-YFP, MCUΔ1-GFP/MICU1-YFP, MCUΔ2-GFP/MICU1-YFP, MCUΔ3-GFP/MICU1-YFP, MCU-GFP/COX8A-RFP and MICU1-YFP/COX8A-RFP. Cells were grown on coverslips, mounted, and subjected to analysis with a Zeiss LSM 510 META confocal microscope. FRET was performed with 100% power bleach iterations (10) of the acceptor using 40 x oil objective. Fluorophores were excited at 488/514, 488/561 and 514/561 nm with photobleaching at 514 or 561 nm. The fluorescence intensities of the donors were recorded at one second intervals. Data were analyzed with the ZEN 2009 software and SigmaPlot11.

Measurement of $[Ca^{2+}]_m$ Uptake and Mitochondrial Membrane Potential ($\Delta\Psi_m$) in Permeabilized Cell System

Control, MICU1-ΔEF1ΔEF2 or MICU1-ΔK mutant HeLa cells were typsinized, counted (6×10^6) and washed in an extracellular-like Ca^{2+}-free buffer (in mM: 120 NaCl, 5 KCl, 1

KH_2PO_4, 0.2 MgCl2, 0.1 EGTA, and 20 HEPES-NaOH, pH 7.4). Following centrifugation, cells were transferred to an intracellular-like medium (protease inhibitors (EDTA-free Complete tablets, Roche Applied Science), 2 μM thapsigargin and 40 μg/ml digitonin. The cell suspension supplemented with succinate (5 mM) was placed in a fluorimeter and permeabilized by gentle stirring. Fura-2FF (0.5 μM) was added at 0 s and JC-1 (800 nm) at 20 s to simultaneously measure extra-mitochondrial Ca^{2+} and $\Delta\Psi_m$. Fluorescence was monitored in a temperature-controlled (37ºC) multi-wavelength-excitation dual wavelength-emission spectrofluorometer (Delta RAM, Photon Technology International) using 490-nm excitation/535-nm emission for the monomer, 570-nm excitation/595-nm emission for the J-aggregate of JC1and 340-nm/380-nm for Fura-2FF. The ratiometric dye, Fura-2FF was calibrated as previously described (Mallilankaraman et al., 2012b). At 450s, 1 μM Ca^{2+} pulse was added and $\Delta\Psi_m$ and extra-mitochondrial Ca^{2+} were monitored simultaneously. CCCP was added at 750 s to determine mitochondrial Ca^{2+} content.

Mitoplast Preparation and Patch-Clamp Recording

Mitoplasts were isolated from HeLa and endothelial cells (10×10^6) which were grown in T-175 flasks, trypsinized, spun at 1200 rpm for 3 minutes, washed with PBS (-Ca^{2+}, -Mg^{2+}), and again spun at 1200 rpm for 3 minutes. For isolation, cells were frozen and thawed to disrupt the cell membrane. Then, the cells were resuspended on ice and homogenized with 30 strokes. The homogenate was centrifuged at 1,000g ruptured and centrifuged. The supernatant was retained. Then, the pellet was resuspended, and again centrifuged. Both supernatants were combined and centrifuged at 12,000g for 15 minutes. The isolated mitochondrial pellet was subjected to mitoplast preparation as described previously (Mallilankaraman et al., 2012a). Freshly isolated mitochondria were suspended in hypotonic solution containing 5 mM sucrose, 5 mM HEPES and 1mM EGTP (pH 7.2). Pelleted mitoplasts were resuspended in 750 mM KCl, 100 mM HEPES and 1 mM EGTA (pH 7.2). Mitochondria and mitoplasts were loaded with the $\Delta\Psi_m$ indicator rhodamine 123 and imaged by confocal microscopy at 488 nm excitation (Carl Zeiss 510 META) or purified mitoplasts were immediately placed on coverslips coated with Cell-Tak (BD Biosciences).

Mitoplast patch clamp recordings were performed at 30°C as detailed previously with modifications (Kirichok et al., 2004). Currents were recorded using a computer controlled Axon200B patch-clamp amplifier with a Digidata 1320A acquisition board (pClamp 10.0 software; Axon Instruments). The ionic composition of the pipette (2, 5 and 100 mM Ca^{2+}) was chosen based on previous measurements. Mitoplasts with p-orbital morphology were used for patch-clamp recording. Mitoplasts were bathed in solution containing sodium gluconate (150 mM), KCl (5.4 mM), $CaCl_2$ (5 mM), Hepes

(10 mM), pH 7.2. The pipette buffer contained sodium gluconate (150 mM), NaCl (5 mM), sucrose (135 mM), HEPES (10 mM) and EGTA (1.5 mM), pH 7.2. After formation of GΩ seals (pipette resistance 20-35 MΩ) the mitoplasts were ruptured with 200-400 mV pulse varied from 2 to 6 ms duration. Mitoplasts capacitance was measured (2.2-3.8 pF). After capacitance compensation, mitoplasts were held at 0 mV and I_{MCU} were elicited with a voltage ramp (from -160 mV to 80 mV, - 120 mV/s).

qRT-PCR Analysis

Total RNA was isolated from cell lines and ECs using RNeasy Mini kit (Qiagen) and total RNA (1 µg) was reverse transcribed with the high capacity cDNA reverse transcription kit (Applied Biosystems ABI 4368814). Quantitative Real-time PCR reactions were performed with gene specific solaris qPCR gene expression assay kit (Abgene® UK) (for human MICU1 and MCU: forward primer: CTTTGACCGAGAGGCTGCT, reverse primer: GTGAGTTCAGACGAAAC and Probe: TGTTTGGACGCGATGTT. Forward primer: GTCAGTTCACACTCAAGCCTAT, reverse primer: TTGAAGCAGCAACGCGAACA, and Probe: TCTATTCACCAGATGGT respectively). The relative gene expression was calculated with neg shRNA in the case of stable knockdown cells, control ECs for CAD/PAD ECs or 293T, EA.hy926 and HBMEC-1 for other cell types using 7300 Real Time PCR system RQ study software 1.4 (Applied Biosystems). The results were expressed as percent mRNA expression and plotted using GraphPad Prism version 5 software.

Detection of Glutathione and Mitochondrial Superoxide Levels

Mitochondrial superoxide was measured using the mitochondrial $O_2^{\bullet-}$ indicator MitoSOX Red (Molecular probes; Invitrogen) as described (Hawkins et al., 2010; Mukhopadhyay et al., 2007). Briefly, neg shRNA, MICU1 KD and MICU1 Rescue EA.hy926 cells grown on coated glass coverslips were loaded with 10 µM monochlorobimane (mBCl) or 5 µM MitoSOX Red for 30 min, coverslips were mounted in an open perfusion microincubator (PDMI-2; Harvard Apparatus) at 37°C and imaged. Confocal (510 Meta; Carl Zeiss, Inc.) images were obtained at 488-nm or 561 nm excitation using a 40x oil objective. Images were analyzed and the mean GSH-MCB and MitoSOX Red fluorescence was quantified using ZEN 2010 software.

Cell Proliferation Assay

To measure EC proliferation, neg shRNA, MICU1 KD and Rescue ECs were stained with 5µM CFSE (Cell Trace™ CFSE Cell proliferation Kit, Molecular Probes, Invitrogen). Briefly ECs were grown on 100 cm^2 dishes to 90% confluence and labeled with 5 µM CFSE for 15 min at 37° C, quenched with pre-warmed complete media for 30 min at

37°C and washed three times with media to remove excessive staining. An equal number of CFSE stained cells were plated and incubated at 37°C, 5% CO_2 for 72 hrs. After 72 hrs, the ECs were washed with PBS and stained with Aqua live dead discrimination dye (Invitrogen). ECs were then fixed with 3.7% paraformaldehyde and the fluorescence intensities acquired using a flow cytometer (LSRII; Becton Dickinson, CA, USA). For flow cytometric analysis a minimum of 100,000 events per sample were collected and the data analyzed using Flow-Jo software. Standard gating procedures using FMO controls were used to identify positive cells/peak. Relative fluorescence intensities were plotted on a logarithmic scale using Flow-Jo software and quantitation was plotted using GraphPad Prism version 5 software.

Endothelial cell Migration Assay

Control, CVD-derived ECs and CVD + MICU1 Rescue ECs were seeded at a density of 1×10^5 cells/well in 6-well plates overnight for confluent monolayer. A uniform 1.8 mm scratch running the entire length of the well was created using a sterile 200 μl tip. The wells were washed three times with PBS to remove the cell debris and then bathed in 2 ml complete endothelial medium. After 24 hrs the cells were washed and fixed with CAMCO Quick Stain® II as per manufacturer instructions. The wells were photographed at multiple locations using phase contrast microscopy with a 4x objective. Migration was quantified using Image J software (NIH); results are expressed as percent gap closure (Craige et al., 2011).

Endothelial-Monocyte Adhesion Assay

Endothelial cells were plated on a glass slide (44 x 20 mm) coated with 0.2% gelatin. ECs were cultured for 24 h. ECs and THP-1 monocytes were labeled with Cell Tracker Green and Cell Tracker Red (1 μM; Invitrogen), respectively for 15 min. Labeled monocytes were evenly added onto ECs and allowed 10 min for adhesion. Unbound monocytes were removed and images were acquired at 488 nm and 561 excitation nm using a 40x oil objective (Hawkins et al., 2007). Adherent monocytes were counted and expressed as percent adhesion (THP-1/ECs). TNF-α (10 ng/ml) or LPS (1 μg/ml) were used as positive controls.

In Vivo Knockdown of MICU1 and Vascular Integrity Measurement

MICU1 knockdown in mice was performed using a lentivirus carrying MICU1-shRNA. Three daily doses of MICU1-shNRA ($1x10^8$ TU/ml as measured by p24 ELISA, and also confirmed by true infectious titer via puromycin resistance in EC culture; 100 μl/mice was delivered using saline as vehicle) lentivirus or control lentivirus neg-shRNA were administered by i.v. and MICU1 expression in the endothelium was assessed by qRT-

PCR after 5 days from the last injection (8 total days). Freshly isolated primary ECs were be rapidly subjected to $[Ca^{2+}]_m$ and ROS measurement. The vascular integrity studies were performed as described previously (Gandhirajan et al., 2013). The animal protocol was approved by Temple University IACUC (#4028).

Statistical analysis

Data from multiple experiments were quantified and expressed as Mean ± SEM., and differences between groups were analyzed by using two-tailed Student's t test. *P* value < 0.05 was considered significant in all analyses. Data were plotted either with Graph pad Prism version 5.0 or with Sigma Plot 11.0 software.

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