SUPPLEMENTAL MATERIAL

Supplemental Methods:

Electrophysiology Study

For the electrophysiology studies, the control mice (n=5), ACE8/8 mice (n=10) and ACE8/8 mice treated with MitoTEMPO (n=6) were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (5 mg/kg). As it was previously described,[1] after cutdown of the right internal jugular vein, a 1.1-F catheter with 0.5-mm inter-electrode spacing (EPR 800, Millar Instruments, Houston, TX) was placed into the vein and was advanced into the right ventricle. A constant current stimulator (A320, World Precision Instruments, Sarasota, FL) connected to a computer was used for cardiac stimulation. During the experiment, body temperature was maintained at 37°C with a warming pad. Burst pacing at cycle lengths of 100 to 30 ms (or to the loss of 1:1 capture) was used to test for VT inducibility. A rhythm with more than three consecutive ventricular beats was considered to be VT.

Telemetry Monitoring

Five untreated ACE8/8 mice and four ACE8/8 mice treated with MitoTEMPO of 4 - 5 weeks of age were implanted with ETA-F10 transmitters (Data Sciences International, St. Paul, M.N.) as we previously described.[1-3] Briefly, mice were anesthetized by intraperitoneal injection of ketamine (100mg/kg) and xylazine (10mg/kg) cocktail. A skin incision was made at right abdominal region and a transmitter was inserted subcutaneously to the left. The two electrocardiogram (ECG) leads were tunneled and positioned under skin to generate a lead II electrocardiographic configuration. The skin incision was then closed, and the animals were followed by telemetry for a maximum of two weeks or until their death. ECG signals were analyzed on the second day after implantation for measurement of basic ECG parameters and ventricular premature beat burden. Both heart rate calculation and cardiac rhythm analysis were performed using Dataquest ART Version 4.1 software (DSI).

Annexin-V Staining

Hearts from three untreated ACE8/8 mice and three ACE8/8 treated with MitoTEMPO were fixed in 10% formalin and sectioned into 8-μm-thick slices. They slices were blocked for one hour at room temperature. Sections were incubated with anti-annexin V antibody (1ug/ml ab14196, Abcam, Cambridge, MA) overnight at 4°C, (1:100 concentration) followed by staining with rabbit IgG secondary antibody. The slides were reviewed using a Zeiss Axioskop microscope (Carl Zeiss Inc, Thornwood, NY).

Mitochondrial ROS Measurement by Confocal Microscopy

To measure mitochondrial ROS, the fluorescent probe MitoSOX Red was used as previously described [4]. Briefly, cardiomyocytes were isolated from control, ACE8/8 or ACE8/8 mice treated with MitoTEMPO (n=3 for each group) as previously described [4]. Cells were stained with 5 μM MitoSOX Red and 100 nM MitoTracker Green for 10 minutes at 37°C, followed by washing twice with warm MEM medium and were incubated for 10 minutes. Images were taken by a Zeiss LSM510 META confocal microscope (Carl Zeiss GmbH, Oberkochen, Germany) using an argon laser excitation (488 nm and 514 nm) with emission collection through a 560-nm long-pass filter. Images were captured using 63× water immersion objective lens at 1024 × 1024 pixels. Cell area was calculated, and the whole-cell fluorescence intensity of MitoSOX Red was measured with ImageJ software (NIH). The number of pixels of the cell fluorescence divided by the cell area was used to determine the mitochondrial ROS generation.

Mitochondrial ROS Measurement by Flow Cytometry

To quantify the mitochondrial ROS by flow cytometry, the measurements were carried out using Cyan ADP (Beckman Coulter, Brea, CA). Isolated cardiomyocytes from each group (n=3 animals for each group) were stained with 5 μM MitoSOX Red with a similar method as above. MitoSOX Red was excited by laser at 488 nm, a similar excitation (514 nm) used in confocal studies, and the data were collected for the FSC, SSC, pulse-width, and 585/42 nm (FL2) channels. Cell debris as represented by distinct, low forward and side scatter were gated out for analysis. The data are presented by histogram of mean intensity of MitoSOX fluorescence or fold change when compared with an unstained control with MitoSOX present.

Transmission Electron Microscopy

Control, ACE8/8 mice, and ACE8/8 mice treated with MitoTEMPO were studied (n=3 for each group). Tissues were washed with cold phosphate buffered saline (PBS), and fixed with EM Grade glutaraldehyde 4% in 0.1M cacodylate buffer (pH 7.4). Fixed tissues were incubated with osmium tetroxide 1% in cacodylate buffer for 2 h and processed for embedding. Ultra-thin sections were cut 83 nm, placed on 200 mesh copper grids, and stained with uranyl acetate and lead citrate. All materials were purchased from Electron Microscopy Sciences (Hatfield, PA). Samples were visualized using a JEM-1220 Jeol transmission electron microscopy (JEM, Peabody, MA), and micrographs were taken using a Gatan Digital Micrograph (Gatan Microscopy, Plesanton, CA). All microscopy measurements were performed using the University of Illinois Central Microscopy Research Core Facility. Random images (n=20) from each sample were taken for analysis. The area occupied by mitochondria and the ratio of the damaged area identified by white blank areas to the area of the whole mitochondrion were measured using a digital grid that was placed over each micrograph (15 x 15 grid with 225 points at cross sections). The points that fell within a mitochondrion were counted and were divided by the total number of points (i.e. 225 minus pseudospaces) to measure the area occupied by

mitochondria. The total points of white areas within a mitochondrion were divided by the total points that fell within the mitochondrion to estimate the damage.

Western Blot Analysis

The control, ACE8/8, and ACE8/8 treated with MitoTEMPO mice (n=5 for each group) were sacrificed, and their hearts were excised. The ventricular tissue was homogenized in a buffer containing 20 mM of tris-(hydroxymethyl)-aminomethane (Tris-Cl) (pH, 7.4), 150 mM of sodium chloride (NaCl), 2.5 mM of ethylenediaminetetraacetic acid (EDTA), 1% Triton-100, 10 μL/mL of phenylmethylsulfonyl fluoride (PMSF), 10 μL/mL of protein inhibitor cocktail (Pierce, Rockford, IL), and 10 μL/mL of phosphatase inhibitor cocktail II (Sigma-Aldrich, St. Louis, MO). Protein samples (5 to 20 μg) were separated via 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to nitrocellulose membranes. The membranes were blotted with the primary antibodies against phosphorylated (Tyr 416) c-Src (at 1:500 concentration) and Cx43 (at 1:3000 concentration) (Cell Signaling, Danvers, MA) at 4°C overnight. For a loading control, the membranes were blotted with a primary antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotech, Santa Cruz, CA). After treatment with secondary anti-rabbit or anti-mouse antibodies (at 1:2000 concentration and 1:10,000 concentration for c-Src and Cx43 respectively with one hour incubation time), imaging was performed with enhanced chemiluminescence. The radiographic film images were scanned and analyzed with NIH ImageJ software. Cx43 and phospho-Src levels were corrected for the GAPDH level for each sample.

Immunohistochemistry

Control, ACE8/8, and ACE8/8 treated with MitoTEMPO mouse hearts (n=4 for each group) were fixed in 10% formalin. After which, 8-μm thick sections were blocked for 1 h at room temperature and then were incubated with anti-Cx43 antibodies (Cell Signaling) overnight at 4°C at concentration of 1:1000. The slides were reviewed with a Zeiss Axioskop microscope (Carl Zeiss, Inc, Thornwood, NY), and photomicrographs with original magnification ×40 were taken from the apex, the mid-left ventricle (LV), and the LV base. From each of those sites, photomicrographs were taken from the endocardium and epicardium. The Cx43 content was quantified with the use of a grid that divided the field of view into 225 squares. At the intersection points aligning with the intercalated disks, Cx43 was scored as "1" (present) or "0" (absent). The results were expressed as the percentage occupied by Cx43 of the total area examined, excluding pseudospaces. This method has been used previously to quantify levels of collagen and Cx43 in cardiac tissue.[1,5,6]

Functional Assessment of Cx43

We used an established technique for measuring Cx43 function that involves fluorescent dye introduction and diffusion in intact heart muscle.[1,7] Fresh hearts from control, ACE8/8 and ACE8/8 mice treated with MitoTEMPO (n=5 for each group) were obtained. A sample from

each heart was placed in phosphate buffered saline at 37°C, the anterior surface of the left ventricle was punctured with a 27-guage needle, and the sample was incubated with a droplet of 0.5% Lucifer yellow (LY) and a droplet of 0.5% Texas Red Dextran (TRD) in 150 mM of LiCl solution. After a 15- minute incubation, the samples were fixed in 4% formaldehyde for 30 min, washed in phosphate-buffered saline, frozen in liquid nitrogen, and sliced into 14-µm sections. The sections were mounted on microscope slides and examined on a Leica DM5000 B epifluorescence microscope (Leica Microsystems Inc., Bannockburn, IL). Digital images of the spread of LY and TRD were obtained. The measurement of the dye spread was performed with ImageJ software. Molecules of TRD are too large to traverse gap junctions and stain cells with disrupted sarcolemmal membranes. The TRD distribution was subtracted from the length of the LY spread at the same site to measure the true LY spread through gap junctions. Dye spread in longitudinal and transverse directions was assessed.

References

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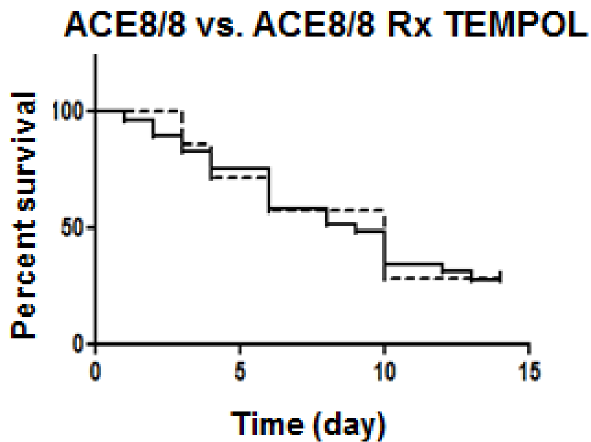
Supplemental Table 1. Basic ECG parameters in untreated and MitoTEMPO treated ACE8/8 mice.

	Premature beats/min	PR (ms)	HR (bpm)	QRSd (ms)	QTc (ms)	Ramp (mV)
ACE8/8	4.42±2.2*	32±1.8	545±52	10±1	69±10	207±110
ACE8/8 Rx MitoTempo	0.75±0.2	41±4	587±63	13±1	85±17	98±45

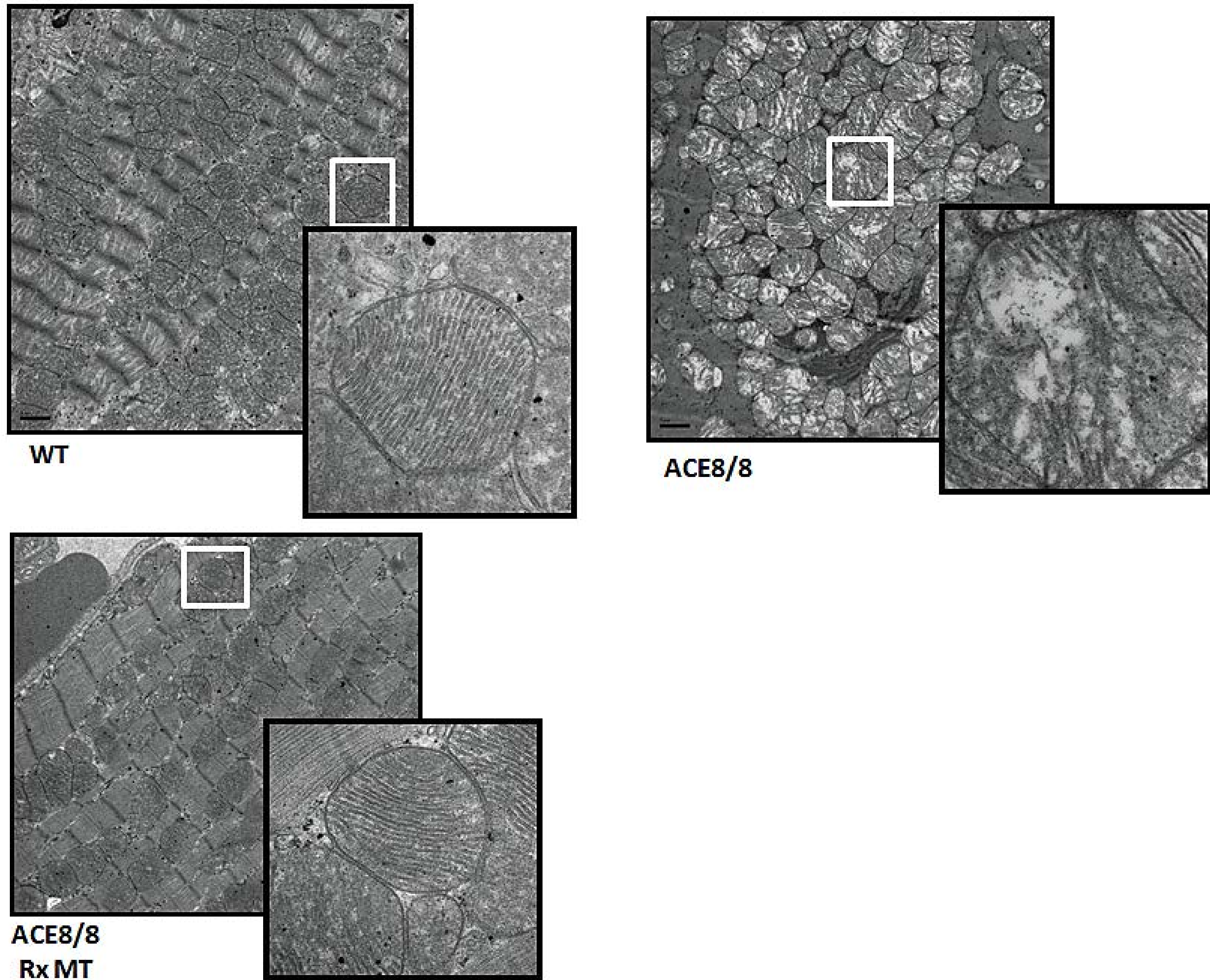
PR, PR interval; HR, heart rate; QRSd, QRS duration; QTc, QT interval corrected for heart rate; Ramp, R wave amplitude; *P value < 0.05

Supplemental Figures:

Supplemental Figure 1. A general antioxidant did not improve survival. Kaplan-Meier survival analysis shows no improvement in the survival from sudden arrhythmic death in ACE8/8 mice treated with TEMPOL.



Supplemental Figure 2. Mitochondrial damage is prevented by MitoTEMPO treatment. Electron microscopic images of cardiomyocyte mitochondria structure in control, ACE8/8 mice and ACE8/8 mice treated with MitoTEMPO.



Supplemental Figure 3. MitoTEMPO prevents activation of c-Src by RAS activation. Western blot analysis shows the level of phospho-(Tyr416) Src (active c-Src) is 32% higher in ACE8/8 than in control mouse hearts ($P<0.05$), and reduces to the level of control after Mito-TEMPO treatment (n= 5 for each group, P=NS).