Mitochondria Oxidative Stress, Connexin43 Remodeling, and Sudden Arrhythmic Death

Ali A. Sovari, MD; Cody A. Rutledge, BS; Euy-Myoung Jeong, PhD; Elena Dolmatova, MD; Divya Arasu, BS; Hong Liu, MD, PhD; Nooshin Vahdani, BS; Lianzhi Gu, MD, PhD; Shadi Zandieh, MD; Lei Xiao, MD, PhD; Marcelo G. Bonini, PhD; Heather S. Duffy, PhD; Samuel C. Dudley Jr, MD, PhD

Background—Previously, we showed that a mouse model (ACE8/8) of cardiac renin–angiotensin system activation has a high rate of spontaneous ventricular tachycardia and sudden cardiac death secondary to a reduction in connexin43 level. Angiotensin-II activation increases reactive oxygen species (ROS) production, and ACE8/8 mice show increased cardiac ROS. We sought to determine the source of ROS and whether ROS played a role in the arrhythmogenesis.

Methods and Results—Wild-type and ACE8/8 mice with and without 2 weeks of treatment with L-NIO (NO synthase inhibitor), sepiapterin (precursor of tetrahydrobiopterin), MitoTEMPO (mitochondria-targeted antioxidant), TEMPO (a general antioxidant), apocynin (nicotinamide adenine dinucleotide phosphate oxidase inhibitor), allopurinol (xanthine oxidase inhibitor), and ACE8/8 crossed with P67 dominant negative mice to inhibit the nicotinamide adenine dinucleotide phosphate oxidase were studied. Western blotting, detection of mitochondrial ROS by MitoSOX Red, electron microscopy, immunohistochemistry, fluorescent dye diffusion technique for functional assessment of connexin43, telemetry monitoring, and in vivo electrophysiology studies were performed. Treatment with MitoTEMPO reduced sudden cardiac death in ACE8/8 mice (from 74% to 18%; P<0.005), decreased spontaneous ventricular premature beats, decreased ventricular tachycardia inducibility (from 90% to 17%; P<0.05), diminished elevated mitochondrial ROS to the control level, prevented structural damage to mitochondria, resulted in 2.6-fold increase in connexin43 level at the gap junctions, and corrected gap junction conduction. None of the other antioxidant therapies prevented ventricular tachycardia and sudden cardiac death in ACE8/8 mice.

Conclusions—Mitochondrial oxidative stress plays a central role in angiotensin II–induced gap junction remodeling and arrhythmia. Mitochondria-targeted antioxidants may be effective antiarrhythmic drugs in cases of renin–angiotensin system activation. (Circ Arrhythm Electrophysiol. 2013;6:623-631.)

Key Words: mitochondria • oxidative stress • sudden cardiac death • ventricular tachycardia

An increased level of angiotensin-II (AngII), as is found in heart failure, is associated with an increased risk of ventricular tachycardia (VT), and treatment with angiotensin-converting enzyme inhibitors and angiotensin receptor blockers reduces that risk.1 Investigating the mechanisms of AngII-induced arrhythmia may result in finding new antiarrhythmic targets. We created a mouse model of cardiac-restricted angiotensin-converting enzyme overexpression. We demonstrated that homozygous mice (ACE8/8) have a high rate of sudden cardiac death (SCD), with telemetry monitoring showing that ≈80% of the SCD resulted from VT and less commonly severe bradycardia and conduction block, in the absence of any left ventricular structural or functional abnormality at the studied age.2 The VT and bradycardia were the result of c-Src tyrosine kinase activation, connexin43 (Cx43) reduction, and the impairment of gap junction conduction.2,3

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AngII is known to increase reactive oxygen species (ROS) levels.4 Excess amounts of ROS have been implicated in the genesis of arrhythmia,2,5,6 and ROS is known to activate c-Src.7 Nevertheless, there is no clear proof that oxidative stress causes arrhythmia or of how oxidative stress might contribute to the arrhythmic substrate. Therefore, we sought to determine whether ROS mediated any of the Cx43 remodeling during renin–angiotensin system (RAS) activation and the principal source of cardiac ROS responsible for arrhythmic risk. Sources of ROS include the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase that generally requires the p67 subunit for activity, xanthine oxidase, uncoupled NO synthase (NOS) in part because of tetrahydrobiopterin depletion,
and mitochondria. We inhibited each source in turn using previously established methods and explored the effect on RAS-induced arrhythmogenesis.

**Materials and Methods**

The animal experiments were performed according to the National Institutes of Health Guide for the Care and Use of Experimental Animals and were approved by the University of Illinois Institutional Animal Care and Use Committee. All mice in this study were started on treatment at the age of 4 weeks, and they were studied for 2 weeks. In our previous studies of ACE8/8 mice, we did not detect any difference in the phenotypes based on the mouse sex. Therefore, we used both sexes for our experiments. A group of wild-type mice (n=10), with similar background to the ACE8/8 mice (C57BL), and the following groups of ACE8/8 mice were studied:

1. ACE8/8 mice untreated control (n=23).
2. ACE8/8 mice treated with 4′-hydroxy-3′methoxyacetophenone (apocynin; Sigma-Aldrich, St. Louis, MO) to inhibit the NADPH oxidase activity (1.5 mmol/L in drinking water for 2 weeks, n=8).
3. ACE8/8 mice crossed with a P67 dominant negative (P67DN) mice to inhibit NADPH oxidase activity (n=10). P67 is an important subunit of NADPH oxidase.
4. ACE8/8 mice treated with N5-(1-iminoethyl)-L-ornithine, dihydrochloride (L-NIO; Sigma-Aldrich) to inhibit NOS (25 mg/kg per day intraperitoneal injections for 2 weeks, n=10). L-NIO is an inhibitor of all NOS subtypes.
5. ACE8/8 mice treated with 2-amino-7,8-dihydro-6-(2S-hydroxy-1-oxopropyl)-4(1H)-pteridinone (sepiapterin; Sigma-Aldrich), a precursor of tetrahydrobiopterin, to prevent eNOS uncoupling without inhibition of NOS (5 mg/kg per day intraperitoneal injections for 2 weeks, n=8).
6. ACE8/8 mice treated with 1,5-dihydro-4H-pyrazolo[3,4-d][pyrimidin-4-one (allopurinol; Sigma-Aldrich), which is a general antioxidant and mimetic of superoxide dismutase (2 mmol/L in drinking water for 2 weeks, n=8).
7. ACE8/8 mice treated with 4-hydroxy-2,2,6,6-tetramethylpiperidinoxy (TEMPOL; Enzo Life Sciences), which is a general antioxidant and a mimetic of superoxide dismutase (2 mmol/L in drinking water for 2 weeks, n=8).
8. ACE8/8 mice treated with (2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl) triphenylphosphonium chloride (MitoTEMPO; Enzo Life Sciences, Plymouth Meeting, PA) to target mitochondrial superoxide (0.7 mg/kg per day intraperitoneal injections for 2 weeks, n=17).

In addition, a group of wild-type mice were treated with MitoTEMPO (0.7 mg/kg per day intraperitoneal injections for 2 weeks, n=5) to evaluate for any possible harmful effects of treatment.

**Figure 1.** A mitochondrial antioxidant inhibits sudden cardiac death and ventricular arrhythmia inducibility. A, Renin–angiotensin system activation mice were treated with the following antioxidants: apocynin, L-NIO, sepiapterin, allopurinol, TEMPOL, and MitoTEMPO. A group of ACE8/8 mice were also crossed with P67DN mice. Kaplan–Meier survival analysis and log-rank tests show significant improvement in the survival free from sudden arrhythmic death only in the ACE8/8 mice that were treated with MitoTEMPO (allopurinol: P=0.49; hazard ratio, 0.75 [confidence interval [CI], 0.28–1.79]; apocynin: P=0.54; hazard ratio, 0.77 [CI, 0.27–1.94]; L-NIO: P=0.9024; hazard ratio, 0.9526 [CI, 0.42–2.16]; P67DN: P=0.22; hazard ratio, 1.77 [CI, 0.74–4.01]; sepiapterin: P=0.67; hazard ratio, 0.83 [CI, 0.31–2.10]). MitoTEMPO had no effect on wild-type mice (WT). B, Representative electrocardiograms (ECG lead II) and right ventricular electrograms (endocardial EGM) of WT, ACE8/8, and ACE8/8 mice treated with MitoTEMPO are shown. Ventricular tachycardia (VT) was induced in 90% of ACE8/8 mice (9 of 10) using a burst pacing protocol starting at 100 ms pacing cycle length (PCL) and decreasing to 30 ms PCL or 2:1 capture. Treatment with MitoTEMPO reduced VT inducibility in ACE8/8 mice to 17% (1 of 6 mice) using the above pacing protocol (P<0.05).
Survival Recording and Analysis
Survival of all treated and untreated groups were recorded every morning during the 2 weeks of treatment and observation. Survival was assessed by using Kaplan–Meier analysis and log-rank tests.

Telemetry Monitoring
Please see Methods in the online-only Data Supplement.

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 studied as previously described (see Methods in the online-only Data Supplement).2

Mitochondrial ROS Measurement by Confocal Microscopy
To measure mitochondrial ROS, the fluorescent probe MitoSOX Red was used as previously described.16 Cardiomyocytes were isolated from control, ACE8/8 mice, or ACE8/8 mice treated with MitoTEMPO (n=3 for each group; see Methods in the online-only Data Supplement).

Mitochondrial ROS Measurement by Flow Cytometry
To quantify the mitochondrial ROS by flow cytometry, the measurements were performed using a CyAn ADP Analyzer (Beckman Coulter, Brea, CA). Isolated cardiomyocytes from each group (n=3 animals for each group) were stained with 5 µmol/L MitoSOX Red with a similar method as above (see Methods in the online-only Data Supplement).

Transmission Electron Microscopy
The control, ACE8/8 mice, and ACE8/8 mice treated with MitoTEMPO were studied (n=3 for each group; see Methods in the online-only Data Supplement).

Western Blot Analysis
The control, ACE8/8, and ACE8/8 mice treated with MitoTEMPO (n=5 for each group) were euthanized, and their hearts were excised (see Methods in the online-only Data Supplement).

Immunohistochemistry
The 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 hour at room temperature and then were incubated with anti-Cx43 antibodies (Cell Signaling) overnight at 4°C at concentrations known to provide the best signal-to-noise ratio. This method has been used previously to quantify levels of collagen and Cx43 in cardiac tissue (see Methods in the online-only Data Supplement).2,17

Functional Assessment of Cx43
We used an established technique for measuring Cx43 function that involves fluorescent dye introduction and diffusion in intact heart muscle (see Methods in the online-only Data Supplement).2,18

Statistical Analysis
The values are presented as the mean±SEM. The t test was used to evaluate the statistical significance between 2 groups for analysis of the mitochondrial ROS measurement, Western blot, immunohistochemistry, and electron microscopy results. One-way ANOVA with post hoc Tukey honestly significant test was used to evaluate the statistical significance among the groups for the analysis of the dye diffusion test. The Fisher exact test for 2×2 tables was used for analysis of the VT inducibility by electrophysiology tests. A P value of <0.05 was reported as statistically significant. The survival data were analyzed with the Kaplan–Meier method, and the P value was calculated with the log-rank test.
Results
Mitochondria-Targeted Antioxidant Therapy Prevented SCD and Inducibility of VT

During 2 weeks of observation with various antioxidants (see the Materials and Methods for details), only treatment with MitoTEMPO prevented SCD and improved survival in the ACE8/8 mice (from 26% to 82%; hazard ratio, 4.8; 95% confidence interval, 2.0–11.5; \( P < 0.005 \)). Although the NADPH oxidase, xanthine oxidase, and uncoupled NOS are potential sources of increased cardiac ROS with RAS activation, none of the other antioxidant therapies prevented SCD (Figure 1A). Treatment with TEMPOL, a general antioxidant that is similar to MitoTEMPO but not targeted to mitochondria, was not associated with improvement of survival free of sudden arrhythmic death (Figure I in the online-only Data Supplement.). Treatment of control mice with MitoTEMPO did not cause any death or gross abnormality in the treated mice. Telemetric monitoring of ACE8/8 (n=5) and ACE8/8 treated with MitoTEMPO (n=4) mice for 2 weeks revealed that all mice that died did so from VT degenerating to ventricular fibrillation. Mice treated with MitoTEMPO had a significantly reduced burden of premature ventricular beats (0.75±0.2 versus 4.4±2.2 premature beats/min; \( P < 0.05 \)) and only untreated mice showed nonsustained VT. Basic ECG parameters were comparable between groups (Table I in the online-only Data Supplement.).

In the in vivo electrophysiology studies, VT was induced in 90% (9 of 10) of ACE8/8 mice using a burst pacing protocol with a mean pacing cycle length of 44 ms. The induced VTs in the ACE8/8 mice were primarily monomorphic (88%). VT inducibility in ACE8/8 mice was decreased from 90% to 17% (1 of 6) by MitoTEMPO treatment (\( P < 0.05 \); Figure 1B). VT could not be induced in control mice.

MitoTEMPO Treatment Reduced Mitochondrial Superoxide Levels

Quantification of mitochondrial ROS levels by the MitoSOX reduction and flow cytometry methods revealed a 1.5-fold increase in the mitochondrial superoxide level in the ACE8/8 mice compared with the control mice (\( P < 0.05 \); Figure 2A). MitoTEMPO treatment reduced mitochondrial ROS level to 1.1-fold of that in the control mice (\( P = 0.45 \); Figure 2A). Quantification of mitochondria by MitoTracker Green did not show any significant change between those groups (Figure 2B).

MitoTEMPO Reversed Mitochondrial Damage in RAS Activation

By electron microscopy, the percentage of the cytoplasmic area occupied by mitochondria was not statistically different among the groups studied, consistent with mitochondria

![Figure 3](http://circep.ahajournals.org/)

Figure 3. Renin–angiotensin system (RAS) activation was associated with mitochondrial injury. Electron microscopy shows damage to the inner membrane and cisterns of mitochondria and vacuous areas within mitochondria areas with RAS activation that are prevented by MitoTEMPO treatment. RAS activation did not significantly change the percentage of area occupied by mitochondria compared with the control (38±2%, 34±5%, 36±4% of cytoplasmic surface area for control, ACE8/8, ACE8/8 treated with MitoTEMPO groups, respectively; \( P = 0.16 \) comparing control with ACE8/8, and \( P = 0.45 \) comparing ACE8/8 with MitoTEMPO), a finding consistent with the MitoTracker Green analysis. MT indicates MitoTEMPO; and WT, wild type.
quantification with MitoTracker Green (Figure 3). Nevertheless, ACE8/8 mice showed significant damage to the mitochondria inner membrane and cisterna (Figure 3; Figure II in the online-only Data Supplement). The damaged area identified by the ratio of vacuous area within a mitochondrion to the whole mitochondrion was significantly higher in the ACE8/8 than in the control mice. This ratio was ameliorated by MitoTEMPO treatment (6.5±3%, 15±4%, and 8.5±3% in the control, the ACE8/8, and the ACE8/8 mice treated with MitoTEMPO, respectively; \( P < 0.05 \) for control compared with ACE8/8 mice). Although mitochondria morphology was improved with 2 weeks of MitoTEMPO treatment, we did not study the time course or durability of the improvements in this study.

Rarely could gap junctions be identified in untreated ACE8/8 cardiomyocytes, but gap junctions could be easily identified in the control and treated groups. Histological analysis and annexin V staining showed no differences in necrosis or apoptosis between treated and untreated groups accompanying these structural changes in mitochondria.

**MitoTEMPO Increased Cx43 Levels at the Gap Junctions**

The total Cx43 level detected by Western blot was decreased in ACE8/8 mice to 24% of control (\( P < 0.05 \)), and MitoTEMPO treatment increased that to 62% of control (\( P < 0.05 \); Figure 4A). By immunohistochemistry, Cx43 level in ACE8/8 mice was decreased at intercalated disks to a comparable level measured by Western blot, and most of the remaining Cx43 in the untreated ACE8/8 mice was no longer located at identifiable intercalated disks (Figure 4B). Cx43 increased significantly after MitoTEMPO treatment (\( P < 0.05 \)). Src is known to displace Cx43 from the intercalated disk,19 and the level of active c-Src, phospho-(Tyr416), was 32% higher in ACE8/8 than in control mouse hearts. Phospho-Src was reduced to that of control mice by MitoTEMPO treatment (\( P = 0.29 \) compared with control; Figure II in the online-only Data Supplement).

**MitoTEMPO Increased Gap Junction Conduction to the Control Level**

To determine whether changes in Cx43 levels resulted in functional changes in gap junction conduction at the whole heart level, an established method of fluorescent dye diffusion was used (Figure 5). The predominant effect of MitoTEMPO on improving the dye diffusion longitudinally was consistent with the improvement of Cx43 level at the gap junctions in immunostaining experiments. The gap junction dye diffusion in the longitudinal direction was reduced in untreated ACE8/8 mice to 62% of that in the control mice (\( P < 0.05 \)). MitoTEMPO treatment returned the gap junction conduction to the normal range (\( P = 0.97 \) compared with control).

**Discussion**

Cx43 is the major structural protein of ventricular gap junctions, and a significant decrease in Cx43 causes sudden arrhythmic death.20 In this study, we showed that cardiac RAS activation, as occurs in heart failure,1,21 was associated with a significant reduction in Cx43. This range of reduction in Cx43...
These experiments establish that AngII-mediated ROS plays a role in ventricular arrhythmogenesis, that the mitochondria are the principal source of ROS leading to the arrhythmic substrate, and that ROS is arrhythmogenic, at least in part, by altering Cx43 probably by ROS activation of c-Src.23,24

Although ROS have been implicated in the genesis of arrhythmia,25–29 translation of those findings to clinical studies using general ROS scavengers, such as vitamin E and C, has not produced impressive results.30 In our study, only a mitochondria-targeted antioxidant was able to prevent arrhythmia. Targeting other known cardiac sources of ROS or using a general antioxidant was ineffective despite dosages and routes of administration that have been shown to be effective in inhibition of the targeted source of ROS production.9–15 This result, particularly the therapeutic difference between TEMPOL and MitoTEMPO treatments, suggests that AngII-mediated ROS production is highly compartmentalized within mitochondria in cardiomyocytes.

It has been recently shown that AngII receptors exist on the mitochondrial inner membrane,31 and that AngII may affect directly mitochondrial ROS production. In addition, an isofrom of the NADPH oxidase (NOX4) exists in mitochondria,32,33 and AngII is known to activate NADPH oxidase.34,35 Although our experiments do not suggest a role for the conventional NADPH oxidase, NOX4 does not require the P67 subunit for its activation,36 and apocynin may not effectively inhibit mitochondrial NOX4-dependent ROS production.37 Therefore, it is possible that this system could be involved in what seems to be AngII signaling directly to mitochondria, possibly through a ROS-induced-ROS mechanism.37

Our study does not preclude the possibility of other sources of ROS contributing to arrhythmogenesis in other cardiac pathological states. The RAS activation model used leaves open the possibility that inhibition of other sources of ROS could be effective in more complicated disease states. Moreover, it has been shown that perfusion of the whole heart or isolated cardiomyocytes with H₂O₂ is arrhythmogenic, which highlights the importance of the amount of ROS production in arrhythmogenesis, independent of the source of ROS.26 Consistent with other reports,5 we found that >30% of the cardiomyocyte area was occupied by mitochondria, and
these mitochondria were producing 1.5× higher superoxide in RAS activation mice than in control mice. Therefore, our results may simply be a function of the relative amounts of the enzymatic ROS sources in a cardiomyocyte. Similar findings of the importance of mitochondria as a source of ROS and accompanying mitochondrial damage were recently reported in other cardiac pathologies, such as heart failure, a RAS activation state.5,38,39

These results may have clinical implications in patients with heart failure because AngII and ROS are elevated in that condition.1,21,40–43 Cx43 is reduced in heart failure, and sudden death is increased.44,45 Our findings collectively can be explained by a signaling cascade where cardiac RAS activation increases mitochondrial ROS production and mitochondrial injury, activates c-Src, reduces Cx43 at intercalated disks through competition with activated c-Src, reduces gap junction function, and increases ventricular arrhythmias (Figure 6). This proposed signaling cascade could explain why angiotensin-converting enzyme inhibitors and AngII receptor blockers decrease sudden death.1,41 In addition, these results may have clinical implications in pathological conditions with elevated levels of ROS and c-Src activation exclusive of AngII, for example, in cardiac ischemia and in ischemia-reperfusion state.19,46

Limitations
Cardiac-restricted elevation of AngII in this model without hypertension, and systolic dysfunction allowed investigation of the direct arrhythmogenesis effects of AngII in the heart. Although it is not generally expected, the results may vary in systemic elevation of AngII. It is also possible that MitoTEMPO exerted part of its antiarrhythmic effects by mechanisms other than c-Src activation and Cx43 remodeling. On the contrary, the lack of ventricular fibrosis, normal cardiac sodium current, and an unchanged ventricular effective refractory period in the ACE8/8 mice at the age they were studied support a major role for mitochondrial ROS in RAS-mediated Cx43 remodeling.2,3,47,48 Although the major effect of MitoTEMPO treatment seemed to increase the amount of Cx43, MitoTEMPO treatment also seemed to increase Cx43 phosphorylation, which may help explain the improvement noted in connexon function. The reason for the early mortality in the MitoTEMPO treated mice is unknown, but it is possible that antioxidant treatment takes several days to reach maximum effect. Because treatments were not continued for >2 weeks, it is unclear whether MitoTEMPO treatment prevented sudden death or simply delayed it.

Conclusions
In summary, we found that RAS activation resulted in mitochondrial injury, mitochondrial ROS production, reduction in Cx43, and increased arrhythmic risk. These changes were ameliorated by a mitochondria-targeted antioxidant but not agents targeted to other sources of cardiac oxidation or a general antioxidant. These results establish that ROS can be arrhythmogenic and elucidate a possible mechanism, whereby ROS can cause arrhythmia.

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Disclosures

Dr Dudley has submitted a patent entitled, “Mitochondrial antioxidants for prevention of sudden death by raising connexin43 levels” based on this work. The other authors have no conflict to report.

References

CLINICAL PERSPECTIVE

Activation of the renin–angiotensin (RAS) system is associated with an increased risk of arrhythmia, and inhibiting the RAS system with angiotensin-converting enzyme inhibitors or angiotensin-II (AngII) receptor blockers reduces that risk. RAS activation is known to increase oxidative stress, and oxidative stress is thought to contribute to the arrhythmic risk. In a mouse model of RAS activation, we showed that the increased arrhythmic risk occurred, at least in part, because AngII signaled a reduction in connexin43, the principal ventricular gap junction protein, allowing conduction between myocytes. Reduced conduction then led to increased risk for arrhythmia. In this study, we show that AngII-induced oxidative stress is critical for the reduction in connexin43, and that oxidative stress arises mainly from mitochondria. A mitochondria-target antioxidant, but not other source-specific or general antioxidants, was able to prevent connexin43 remodeling and arrhythmias. Therefore, mitochondrial oxidative stress plays a central role in AngII-induced gap junction remodeling and arrhythmia. These results may have clinical implications in patients with heart failure because AngII and ROS are elevated in that condition, connexin43 is reduced, and sudden death is increased. Our observations could explain why angiotensin-converting enzyme inhibitors and AngII receptor blockers decrease sudden death in heart failure. Moreover, mitochondria-targeted antioxidants may be effective antiarrhythmic drugs in cases of RAS activation, such as heart failure, and may represent a new paradigm of raising channel levels to ameliorate arrhythmic risk.
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, 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. 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. Briefly, cardiomyocytes were isolated from control, ACE8/8 or ACE8/8 mice treated with MitoTEMPO (n=3 for each group) as previously described. 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, Pleasanton, 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


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

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

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).