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(54) **METHOD FOR AMELIORATING OR PREVENTING ARRHYTHMIC RISK ASSOCIATED WITH CARDIOMYOPATHY**

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(52) **U.S. Cl.** ..... **424/94.1**; 514/44 R; 514/89

(57) **ABSTRACT**

A method for reducing arrhythmic risk associated with cardiomyopathy includes administering a composition containing NAD^+ or a mitochondrial targeted antioxidant to an individual in need thereof.

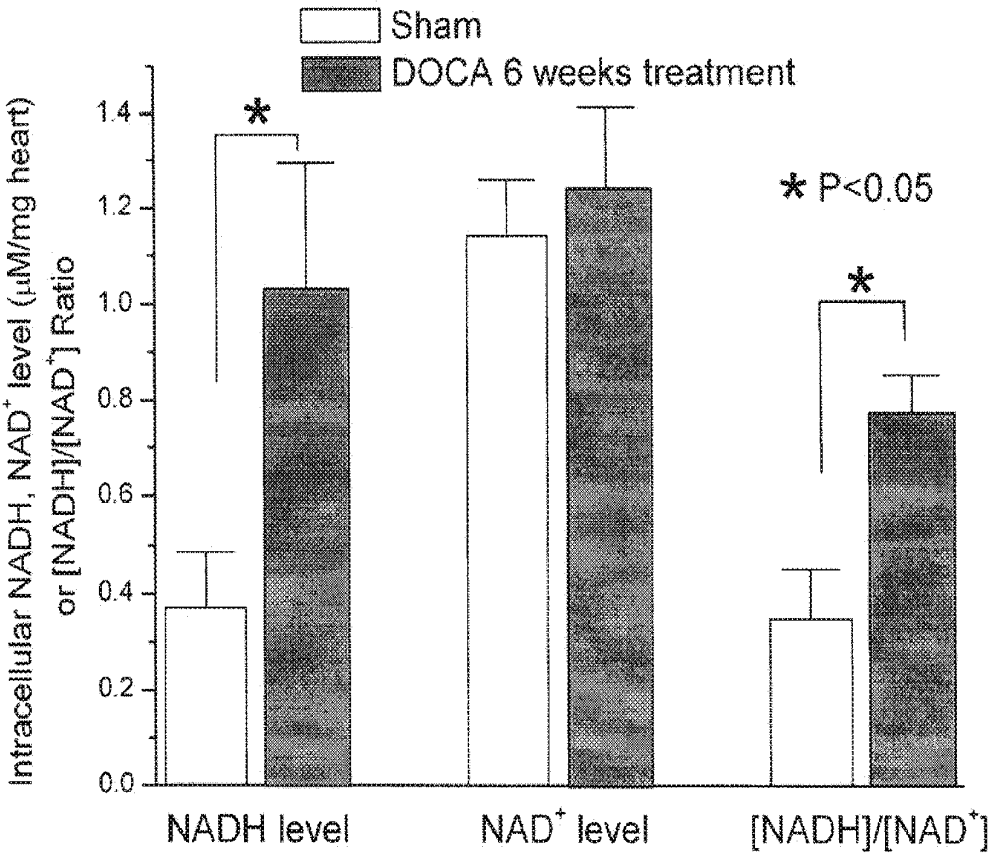
Sham
DOCA 6 weeks treatment
Intracellular NADH, NAD+ level (μM/mg heart) or [NADH]/[NAD+] Ratio
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
*
*
* P<0.05
NADH level
NAD+ level
[NADH]/[NAD+]

FIG. 1

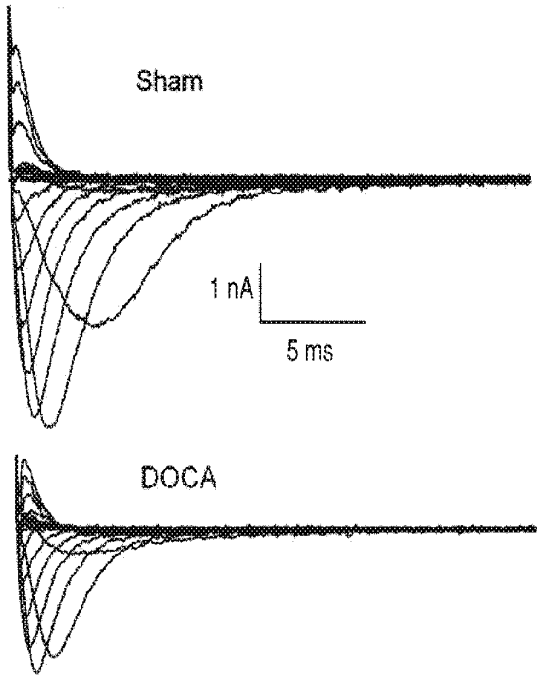
Sham
1 nA
5 ms
DOCA

FIG. 2A

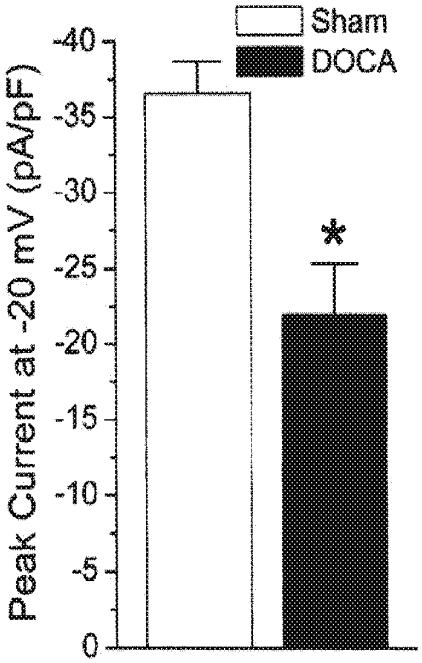
Sham
DOCA
Peak Current at -20 mV (pA/pF)
-40
-35
-30
-25
-20
-15
-10
-5
0
*

FIG. 2B

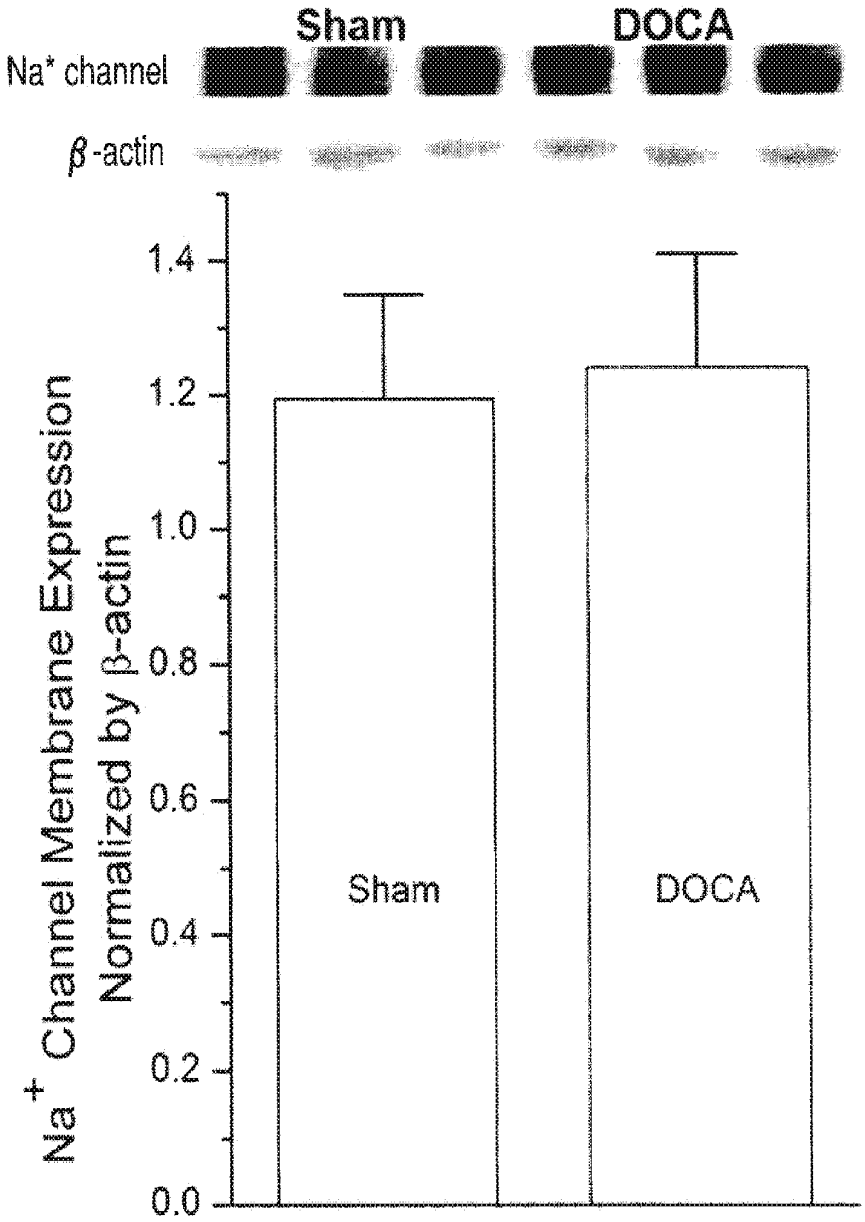
Sham
DOCA
Na* channel
β-actin
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Na+ Channel Membrane Expression Normalized by β-actin
Sham
DOCA

FIG. 3

FIG. 4A

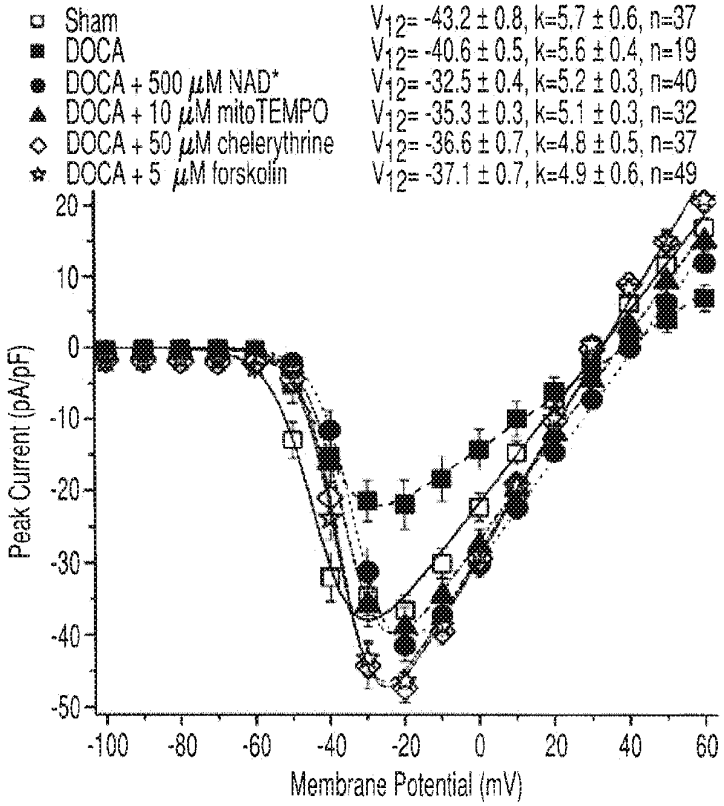
Sham
DOCA
DOCA + 500 μM NAD+
DOCA + 10 μM mitoTEMPO
DOCA + 50 μM chelerythrine
DOCA + 5 μM forskolin
V12= -43.2 ± 0.8, k=5.7 ± 0.6, n=37
V12= -40.6 ± 0.5, k=5.6 ± 0.4, n=19
V12= -32.5 ± 0.4, k=5.2 ± 0.3, n=40
V12= -35.3 ± 0.3, k=5.1 ± 0.3, n=32
V12= -36.6 ± 0.7, k=4.8 ± 0.5, n=37
V12= -37.1 ± 0.7, k=4.9 ± 0.6, n=49
Peak Current (pA/pF)
Membrane Potential (mV)

FIG. 4B

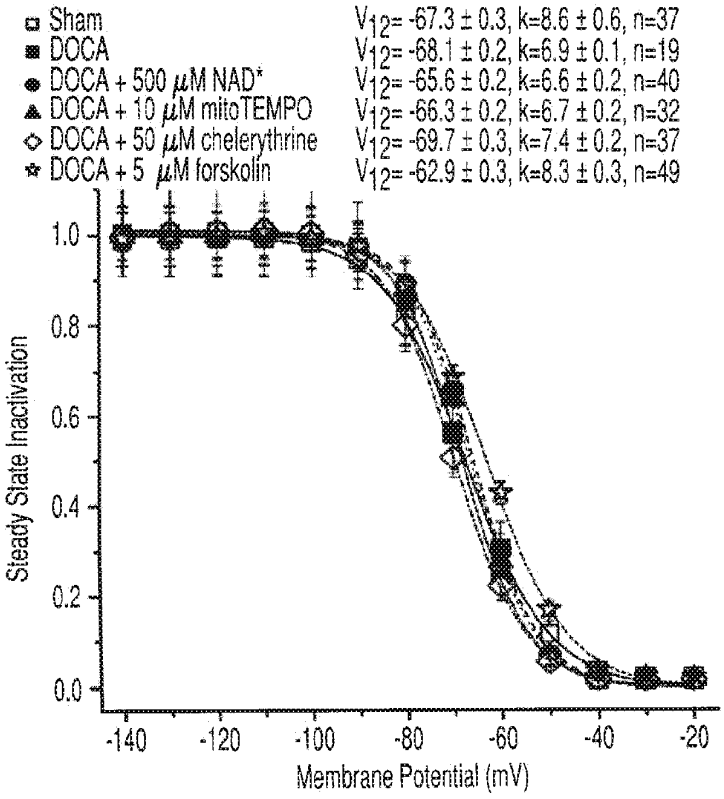
Sham
DOCA
DOCA + 500 μM NAD+
DOCA + 10 μM mitoTEMPO
DOCA + 50 μM chelerythrine
DOCA + 5 μM forskolin
V12= -67.3 ± 0.3, k=8.6 ± 0.6, n=37
V12= -68.1 ± 0.2, k=6.9 ± 0.1, n=19
V12= -65.6 ± 0.2, k=6.6 ± 0.2, n=40
V12= -66.3 ± 0.2, k=6.7 ± 0.2, n=32
V12= -69.7 ± 0.3, k=7.4 ± 0.2, n=37
V12= -62.9 ± 0.3, k=8.3 ± 0.3, n=49
Steady State Inactivation
Membrane Potential (mV)

FIG. 4C

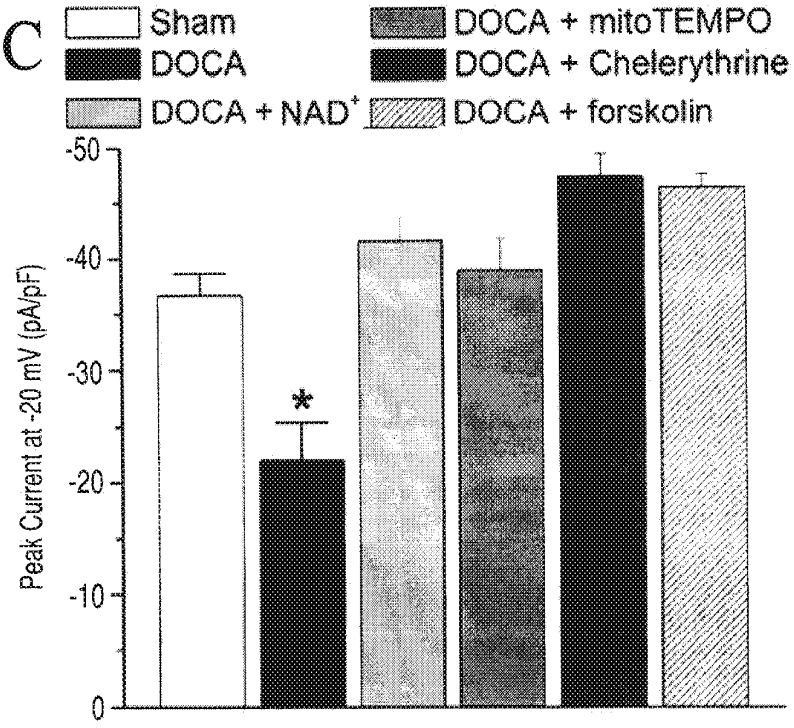
Sham
DOCA
DOCA + NAD+
DOCA + mitoTEMPO
DOCA + Chelerythrine
DOCA + forskolin
Peak Current at -20 mV (pA/pF)
-50
-40
-30
-20
-10
0
*

FIG. 4D

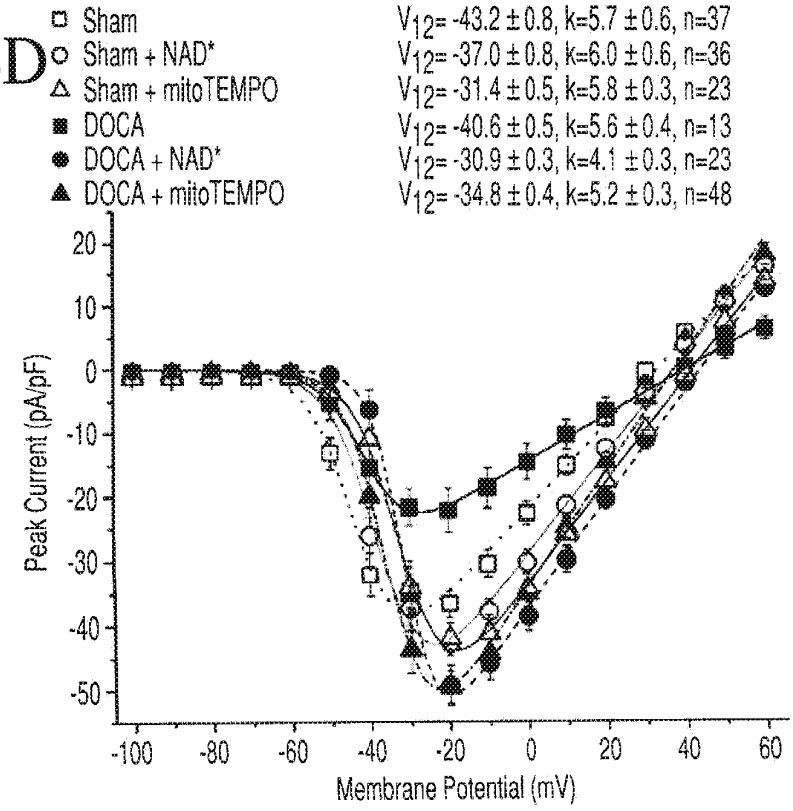
Sham
Sham + NAD+
Sham + mitoTEMPO
DOCA
DOCA + NAD+
DOCA + mitoTEMPO
V12= -43.2 ± 0.8, k=5.7 ± 0.6, n=37
V12= -37.0 ± 0.8, k=6.0 ± 0.6, n=36
V12= -31.4 ± 0.5, k=5.8 ± 0.3, n=23
V12= -40.6 ± 0.5, k=5.6 ± 0.4, n=13
V12= -30.9 ± 0.3, k=4.1 ± 0.3, n=23
V12= -34.8 ± 0.4, k=5.2 ± 0.3, n=48
Peak Current (pA/pF)
20
10
0
-10
-20
-30
-40
-50
-100
-80
-60
-40
-20
0
20
40
60
Membrane Potential (mV)

FIG. 4E

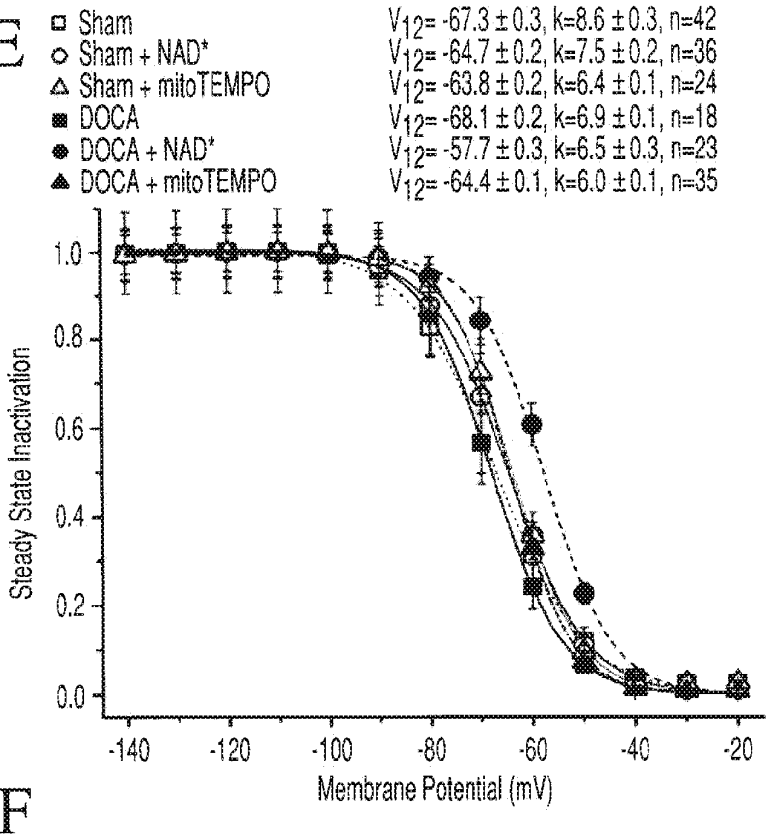
Sham
Sham + NAD+
Sham + mitoTEMPO
DOCA
DOCA + NAD+
DOCA + mitoTEMPO
V12= -67.3 ± 0.3, k=8.6 ± 0.3, n=42
V12= -64.7 ± 0.2, k=7.5 ± 0.2, n=36
V12= -63.8 ± 0.2, k=6.4 ± 0.1, n=24
V12= -68.1 ± 0.2, k=6.9 ± 0.1, n=18
V12= -57.7 ± 0.3, k=6.5 ± 0.3, n=23
V12= -64.4 ± 0.1, k=6.0 ± 0.1, n=35
Steady State Inactivation
1.0
0.8
0.6
0.4
0.2
0.0
-140
-120
-100
-80
-60
-40
-20
Membrane Potential (mV)

FIG. 4F

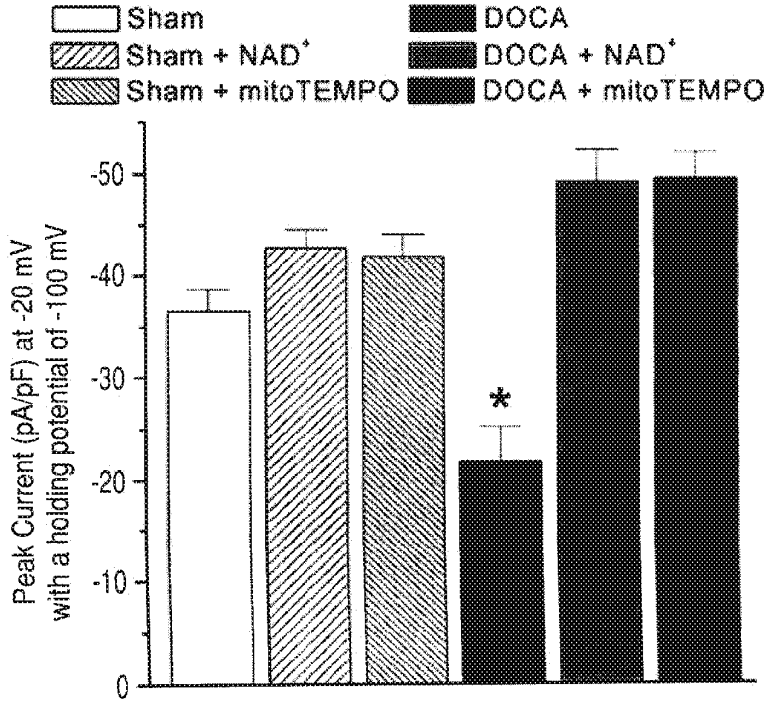
Sham
Sham + NAD+
Sham + mitoTEMPO
DOCA
DOCA + NAD+
DOCA + mitoTEMPO
Peak Current (pA/pF) at -20 mV with a holding potential of -100 mV
-50
-40
-30
-20
-10
0
*

FIG. 5A

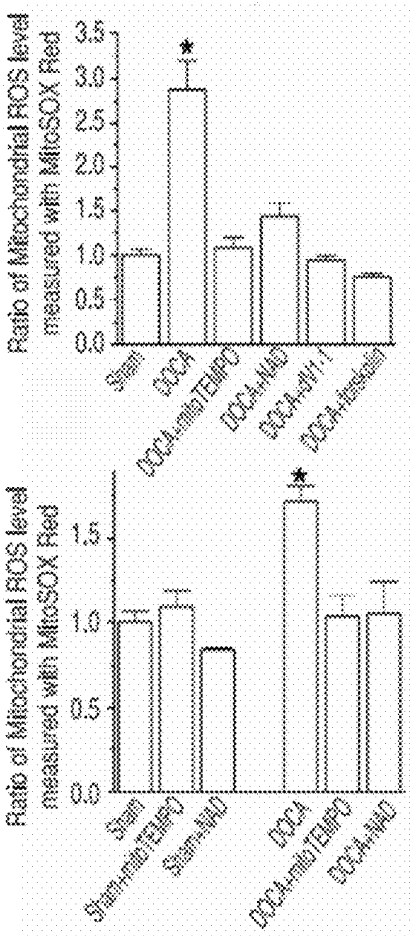

FIG. 5B

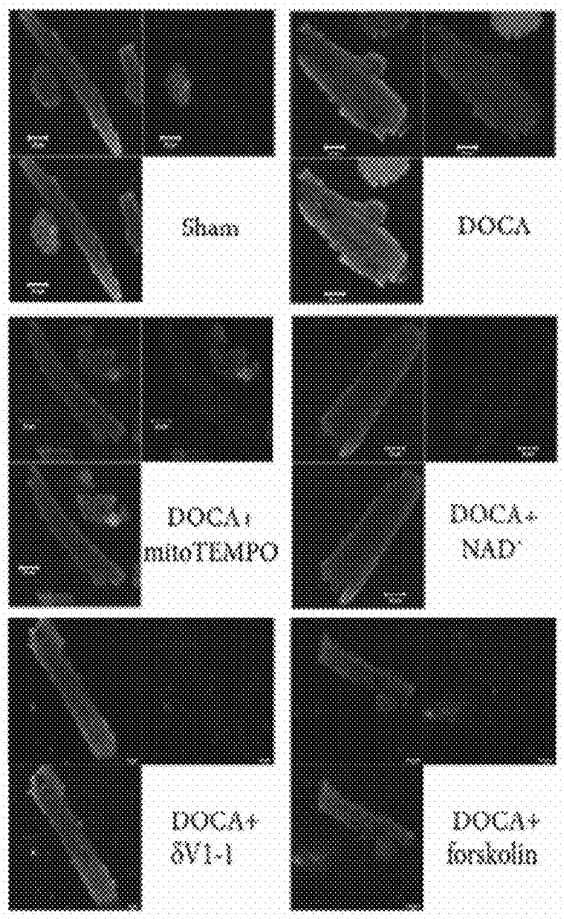

FIG. 5C

METHOD FOR AMELIORATING OR PREVENTING ARRHYTHMIC RISK ASSOCIATED WITH CARDIOMYOPATHY

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This is a continuation-in-part (CIP) application of U.S. application Ser. No. 12/929,786, filed Feb. 16, 2011, which claims the priority benefit of U.S. Provisional Patent Application Ser. No. 61/305,668, filed Feb. 18, 2010, and is a continuation-in-part (CIP) application of U.S. application Ser. No. 12/289,005, filed Oct. 17, 2008, now U.S. Pat. No. 8,003,324B2, which claims the priority benefit of U.S. Provisional Patent Application Ser. No. 60/960,883, filed Oct. 18, 2007, all of the foregoing are hereby incorporated herein in their entirety by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] The invention was made with government supported under grants NIH R01 HL085558, R01 HL072742, R01 HL106592, R01 HL104025, T32 HL072742, and P01 HL058000 (SCD), and a VA MERIT grant. The government has certain rights in the invention.

FIELD AND BACKGROUND OF THE INVENTION

[0003] The present invention is generally directed to cardiac therapy, and more particularly to ameliorating, preventing, and/or reversing arrhythmic risk associated with cardiomyopathy.

[0004] Despite extensive research and novel treatments, conditions associated with deranged cardiac metabolism, such as heart failure or ischemia, are still accompanied by a substantial risk of arrhythmic sudden death (Reference 1). While implanted cardiac defibrillators have decreased sudden death risk, they can cause physical and psychological complications. They are also expensive, and do not address the underlying pathology that leads to arrhythmic risk (References 2-3). A more complete molecular understanding of the basis for the increased arrhythmic risk is likely to lead to new therapies that will be more effective and less invasive.

[0005] Cardiac injury from many causes is associated with altered metabolism and downregulation of the cardiac Na^+ channel (Na_v 1.5) (References 4-7). Recently, we reported that an elevation of intracellular reduced nicotinamide adenine dinucleotide (NADH) can downregulate Na^+ current (I_{Na}) acutely and to a degree that is large enough to be clinically significant (Reference 8). The signaling cascade involves a protein kinase C (PKC)-mediated increase in mitochondrial reactive oxygen species (ROS) production (References 9-10). NADH is known to oscillate with myocardial ischemia, and mitochondrial injury is associated with increased NADH and ROS levels (References 11-12). These changes could contribute to reduced I_{Na}, conduction block, and arrhythmic risk known to exist with reduced cardiac contractility. The NADH effect on ROS production and I_{Na} can be antagonized by PKA activation mediated by NAD^+, by superoxide dismutase, or by 2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (MitoTEMPO), a scavenger of mitochondrial ROS (References 9-10).

[0006] In this study, we tested whether NADH and mitochondrial ROS were elevated in nonischemic cardiomyopathy and whether these changes resulted in a reduction in I_{Na}. We also investigated whether NAD^+ and 2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (MitoTEMPO) could counteract the effects of NADH on mitochondrial ROS and cardiac I_{Na} in nonischemic cardiomyopathy. The PKC inhibitor chelerythrine and PKA activator forskolin were utilized to test whether they participated in the signaling pathway of NADH/NAD^+ modification of the Na_v 1.5 of DOCA myocytes.

ASPECTS OF THE INVENTION

[0007] The present disclosure is directed to various aspects of the present invention.

[0008] One aspect of the present invention includes discovery and/or demonstration that mitochondria are the main source of NADH-dependent ROS downregulating sodium channel current (I_{Na}) in cardiomyopathic cells.

[0009] Another aspect of the present invention includes discovery and/or demonstration that mitochondrial superoxide release is responsible for downregulation of I_{Na} in cardiomyopathic cells.

[0010] Another aspect of the present invention includes discovery and/or demonstration that elevation in intracellular NADH results in activation of protein kinase C (PKE) and subsequent mitochondrial complex III release of reactive oxygen species (ROS) through the mitochondrial inner member anion channel (IMAC) in cardiomyopathic cells.

[0011] Another aspect of the present invention includes discovery and/or demonstration that inhibition of mitochondrial ROS overproduction by one or more strategies prevents or suppresses I_{Na} downregulation by NADH in cardiomyopathic cells.

[0012] Another aspect of the present invention includes suggestions and/or development of possible therapeutic approaches or strategies to reduce or prevent arrhythmic risk associated with cardiomyopathy.

[0013] Another aspect of the present invention includes a method for reducing arrhythmic risk associated with cardiomyopathy, including the step of administering a composition containing NAD^+ to an individual in need thereof.

[0014] Another aspect of the present invention includes a method for restoring the cardiac sodium current to a normal level in an individual with cardiomyopathy, including the step of administering a composition containing NAD^+ to an individual in need thereof.

[0015] Another aspect of the present invention includes a method for reducing arrhythmic risk in an individual with cardiomyopathy and a cardiac ejection fraction of less than 50%, including the step of administering a composition containing NAD^+ to an individual in need thereof.

[0016] Another aspect of the present invention includes a method for reducing arrhythmic risk associated with cardiomyopathy, including the step of administering a mitochondrial targeted antioxidant to an individual in need thereof.

[0017] Another aspect of the present invention includes a method for restoring the cardiac sodium current to a normal level in an individual with cardiomyopathy, including the step of administering a mitochondrial targeted antioxidant to an individual in need thereof.

[0018] Another aspect of the present invention includes a method for reducing arrhythmic risk in an individual with cardiomyopathy and a cardiac ejection fraction of less than

50%, including the step of administering a mitochondrial targeted antioxidant to an individual in need thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0020] One of the above and other aspects, novel features and advantages of the present invention will become apparent from the following detailed description of the non-limiting preferred embodiment(s) of invention, illustrated in the accompanying drawings, wherein:

[0021] FIG. **1** illustrates intracellular NADH and NAD^+ levels and the $[NADH]_i/[NAD^+]_i$ ratio measured in sham and DOCA cardiomyopathic heart tissue. Increased NADH level and $[NADH]_i/[NAD^+]_i$ ratio were seen in DOCA mice;

[0022] FIGS. **2**A-B illustrate that decreased I_{Na} was seen in DOCA cardiomyopathic mice;

[0023] FIG. **2**A illustrates representative whole cell current traces of I_{Na} from sham and DOCA mouse ventricular cardiomyocytes held at −100 mV and measured from −100 to +60 mV with 10 mV steps;

[0024] FIG. **2**B illustrates peak I_{Na} from sham and DOCA mice ventricular cardiomyocytes measured at −20 mV. *P<0.01;

[0025] FIG. **3** illustrates Na_v 1.5 membrane expression measured with biotinylation as unchanged between sham and DOCA mice. In these Western blots, β-actin was used as a loading control. There is no significant change of Na_v 1.5 protein membrane expressions in DOCA mice cardiomyocytes;

[0026] FIGS. **4**A-F illustrate that reduced I_{Na} in cardiomyopathy was corrected by NAD^+ or 2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (MitoTEMPO) without significant changes in channel gating;

[0027] FIG. **4**A illustrates the peak current-voltage relationship;

[0028] FIG. **4**B illustrates the voltage dependence of steady state inactivation of isolated cardiomyocytes from sham and DOCA mouse model treated with NAD^+, 2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (MitoTEMPO), chelerythrine, or forskolin. The minor shifts of $V_{1/2}$ values of steady state gating were not enough to affect the evaluation of the peak currents;

[0029] FIG. **4**C illustrates application of NAD^+, 2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl) triphenylphosphonium chloride (MitoTEMPO), chelerythrine, or forskolin (500, 10, 50, or 5 μM, respectively) intracellularly to isolated cardiomyocytes of DOCA mouse model restored the decreased peak I_{Na} in cardiomyopathic DOCA myocytes at −20 mV. *P<0.01 vs sham group;

[0030] FIG. **4**D illustrates the peak current-voltage relationship of isolated cardiomyocytes from sham and DOCA mouse model with injection of NAD^+ or 2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (MitoTEMPO) (100 or 0.7 mg/kg, respectively). The minor shifts of $V_{1/2}$ values of steady state gating were not enough to affect the evaluation of the peak currents;

[0031] FIG. **4**E illustrates the voltage dependence of steady state inactivation of isolated cardiomyocytes from sham and DOCA mouse model with injection of NAD^+ or 2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (MitoTEMPO) (100 or 0.7 mg/kg, respectively). The minor shifts of $V_{1/2}$ values of steady state gating were not enough to affect the evaluation of the peak currents;

[0032] FIG. **4**F illustrates DOCA mice injected with NAD^+ or 2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (MitoTEMPO) (100 or 0.7 mg/kg, respectively) showed recovered I_{Na}. *P<0.01 vs sham group;

[0033] FIGS. **5**A-C illustrate that mitochondrial ROS levels were increased in DOCA myopathic mice and reduced by NAD^+, 2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (MitoTEMPO), δV1-1 and forskolin;

[0034] FIG. **5**A illustrates mitochondrial ROS overproduction was observed with DOCA mice myocytes by MitoSOX™ Red (2.9±0.3-fold of sham, P<0.01). DOCA cardiomyocyte treatment with NAD^+, 2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl) triphenylphosphonium chloride (MitoTEMPO), δV1-1, or forskolin (500, 10, 50, or 5 μmol/L, respectively) extracellularly decreased ROS levels in myopathic mouse myocytes similar to the sham group (1.4±0.1, 1.1±0.1, 0.9±0.1, or 0.8±0.1-fold of sham, respectively, P>0.05). Three to five animals were tested in each group, and total 29-43 cells were used for average;

[0035] FIG. **5**B illustrates DOCA mice injected with NAD^+ (100 mg/kg) or 2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (MitoTEMPO) (0.7 mg/kg) had decreased mitochondrial ROS (1.1±0.1- or 1.1±0.2-fold of sham, respectively, P>0.05). Three to four animals were tested in each group; and

[0036] FIG. **5**C illustrates representative images from confocal microscopy obtained before and after treatment of myocytes in vitro, labeled with MitoTracker™ Green and MitoSOX™ Red. The white scale bar is 20

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT(S) OF THE INVENTION

[0037] Cardiomyopathy is associated with cardiac Na^+ channel downregulation that may contribute to arrhythmias. Previously, we have shown that elevated intracellular NADH causes a decrease in cardiac Na^+ current (I_{Na}) signaled by an increase in mitochondrial reactive oxygen species (ROS). The decrease in I_N, can be ameliorated by NAD^+ or a mitochondrial specific antioxidant, 2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (MitoTEMPO).

[0038] Here, we tested whether the NADH-mitochondrial ROS pathway was involved in the reduction in I_{Na} in a nonischemic cardiomyopathic model.

Materials and Methods

Model Generation and Isolation of Mice Ventricular Myocytes

[0039] Nonischemic cardiomyopathy was induced in C57BL/6 mice by six weeks of hypertension evoked after unilateral nephrectomy, deoxycorticosterone acetate (DOCA) pellet implantation, and 1% salt water substitution

(Reference 13). Sham operated mice were used as controls. Ketamine (100 mg/kg) and xylazine (10 mg/kg) were administrated by IP pre-operation and buprenorphine (0.1 mg/kg) was injected subcutaneously post-operation and at 12-hour interval as needed. For each experiment, three to eight mice were used.

[0040] Ventricular myocytes were isolated as described before (References 13-14). Briefly, hearts were excised from anesthetized mice, perfused with perfusion buffer (in mmol/L: NaCl 113, KCl 4.7, Na_2HPO_4 0.6, KH_2PO_4 0.6, $MgSO_4$ 1.2, Phenol Red 0.032, $NaHCO_3$ 12, $KHCO_3$ 10, HEPES 10, Taurine 30, 2-3-butanedione monoxime 10) and digested with collagenase II (Worthington Biochemical Co. Lakewood, N.J.). Cardiomyocytes were washed with control buffers (in mmol/L: NaCl 133.5, KCl 4, Na_2HPO_4 1.2, HEPES 10, $MgSO_4$ 1.2) with serially increasing Ca^{2+} concentrations (0.2, 0.5, and 1 mmol/L). Then, myocytes were incubated in MEM medium (modified Eagle's medium with 1% insulin-transferrin-selenium, 0.1% bovine serum albumin, 1% glucose, and 1% penicillin/streptomycin) in a 95% O_2/5% CO_2 incubator at 37° C. for 2 hours prior to being used for patch clamp recording and ROS level measurements.

Documentation of Cardiomyopathy

[0041] Blood pressure and heart rate were measured on acclimated conscious mice six weeks after surgery using tail-cuff plethysmography (Columbus Instruments, Columbus, Ohio). Transthoracic echocardiography was performed using the Vevo 770 system equipped with a RMV-707B transducer (VisualSonics, Toronto, Canada). Mice were anesthetized with 1% isoflurane in oxygen and were closely monitored during the procedure. Images were obtained from the parasternal long axis view and parasternal short axis view at the midpapillary level. Wall thickness, chamber size, fractional shortening (% FS), and ejection fraction (% EF) were evaluated by two-dimensional and M-mode echocardiography. Measurements were averaged from three consecutive beats.

Intracellular NADH and NAD^+ Levels

[0042] Intracellular NADH and NAD^+ levels ($[NADH]_i$ and $[NAD^+]_i$) were detected using the EnzyChrom™ NAD^+/NADH Assay Kit (BioAssay Systems, Hayward, Calif.) with sham and DOCA mice heart tissue followed the manufacturer's instructions. The intensity difference of the reduced product color, measured at 565 nm at time zero and 15 min later, was proportional to the change in the concentration of NAD(H).

Cellular Electrophysiology

[0043] Na^+ currents were measured using the whole-cell patch clamp technique in voltage-clamp mode at room temperature (References 9-10). To measure Na^+ currents, pipettes (1-2 MΩ) were filled with a pipette solution containing (in mmol/L): CsCl 80, cesium aspartate 80, EGTA 11, $MgCl_2$ 1, $CaCl_2$ 1, HEPES 10, and Na_2ATP 5 (adjusted to pH 7.4 with CsOH). The bath solution consisted of (in mmol/L): NaCl 15, CsCl 5, $CaCl_2$ 1, $MgCl_2$ 1, tetramethylammonium Cl 20, N-methyl-D-glucamine 100, 4-aminopyride 3, $MnCl_2$ 2, HEPES 10 and glucose 10 (adjusted to pH 7.4 with CsOH). A stepped voltage protocol from −100 to +60 mV with a holding potential of −100 mV was applied to establish the presence of Na_v 1.5. Peak currents obtained during steps to −20 mV were used for comparison in determining the relative reduction of I_{Na}. To minimize time-dependent drift in gating parameters, all protocols were initiated 2-5 min after whole-cell configuration was obtained. The currents were normalized with cell capacitance.

Measurement of Mitochondrial ROS

[0044] To measure mitochondrial ROS, the fluorescent probe MitoSOX™ Red was used according to the manufacturer's protocol. Briefly, eight groups of isolated cardiomyocytes were studied: sham mouse myocytes, DOCA mouse myocytes, myocytes from sham or DOCA mice treated with 500 μmol/L NAD^+, 10 μmol/L 2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (MitoTEMPO), 50 μmol/L chelerythrine, or 5 μmol/L forskolin for 10 min at room temperature, and myocytes from sham or DOCA mice injected with 100 mg/kg NAD^+ or with 0.7 mg/kg 2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (MitoTEMPO) twice (at 24 h and 1 h before myocyte isolation, respectively). Cells were incubated with 5 μmol/L MitoSOX™ Red and 100 nmol/L MitoTracker Green for 10 min at 37° C., followed by washing three times with MEM medium. Images were taken on a Zeiss LSM710 confocal microscope (Carl Zeiss GmbH, Oberkochen, Germany) using an argon laser excitation (514 nm) with emission collection through a 560 nm long pass filter. The mean values of the whole cell fluorescence of MitoSOX™ Red were obtained with ImageJ software.

[0045] For flow cytometry measurements, isolated cardiomyocytes from sham or DOCA with/without treatments were incubated with MitoSOX™ Red (5 μmol/L) for 15 min and washed twice with media. Appropriate gating was used to select cardiomyocytes, and ~10,000 cells were read in each sample at FL-2 in CyAN ADP flow cytometry (Beckman-Coulter, Brea, Calif.).

Biotinylation and Western Blotting of Na_v 1.5

[0046] Biotinylation with the Pierce® Cell Surface Protein Isolation Kit (Pierce Biotechnology, Rockford, Ill.) and analysis Na^+ channels present at the cell surface were performed on freshly isolated cardiomyocytes of sham and DOCA mice as previously described (Reference 15). For detection of Na_v 1.5, the primary antibody (rabbit anti-SCN5A, Alomone Labs, Jerusalem, Israel) was diluted 1:200. Horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Cell Signaling Technology, Danvers, Mass.) was diluted 1:5000. Actin (Santa Cruz Biotechnology, CA) was used as a loading control.

SCN5A RNA Abundance

[0047] Total RNA was isolated (RNeasy Minikit—Qiagen, Valencia, Calif.) from snap frozen ventricular tissue samples taken from sham and DOCA mice (n=3 per group). Equal quantities of total RNA from all samples were used to generate cDNA using the High Capacity cDNA synthesis kit (Applied Biosystems, Carlsbad, Calif.), and quantitative PCR was performed using Fast SYBR green chemistry (Applied Biosystems, Carlsbad, Calif.) on an ABI 7500 platform. Primers were designed against mouse SCN5A (SCN5A_F TTGCTCCTTCTCTCATGGTTG and SCN5A_R CATGGAGATGCTCAAGAAGGA) and Hypoxanthine phosphoribosyltransferase (HPRT) (HPRT_F AGGCCAGACTTTGT-

TGGATTT and HPRT_R GGCTTTGTATTTGGCTTTTCC) using Primer3 plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus) and synthesized by MWG (Huntsville, Ala.). HPRT acted as the housekeeping gene by which to normalize SCN5A cDNA. The $2^{-\Delta\Delta Ct}$ method was used for relative quantification between groups. A t-test was used for test for statistical comparison between the two groups.

Statistical Evaluations

[0048] Data are shown as the mean±SEM. Aside from above, determinations of statistical significance were performed with ANOVA with the Bonferroni correction for comparisons of multiple means. A value of $P<0.05$ was considered statistically significant.

Results

[0049] At 6 weeks after surgery, DOCA mice had developed hypertension by tail-cuff blood pressure measurements and systolic heart dysfunction by echocardiography (Table 1). Compared to the sham mice, DOCA mice showed higher artery blood pressure, enlarged left ventricular chamber, and reduced ejection fraction ($P<0.05$).

TABLE 1

Blood pressure and Echocardiographic Comparison Between DOCA and Sham Mice

	Sham		DOCA		
	Value	N	Value	N	P value
Heart rate (bpm)	528 ± 17	4	533 ± 28	5	NS
SBP (mmHg)	99 ± 7	4	116 ± 3	5	<0.05
DBP (mmHg)	74 ± 5	4	89 ± 3	5	<0.05
LVESV (μL)	42.1 ± 3.6	8	64.6 ± 3.3	8	<0.05
LVEDV (μL)	87.9 ± 6.1	8	104.7 ± 3.9	8	<0.05
FS (%)	26.4 ± 1.0	8	17.9 ± 1.0	8	<0.05
EF (%)	49.4 ± 3.7	8	37.1 ± 1.8	8	<0.05

Note:

SBP: systolic artery blood pressure; DBP: diastolic artery blood pressure; LVESV: left ventricular end-systolic volume; LVEDV: left ventricular end-diastolic volume; FS: fractional shortening; EF: ejection fraction. Values were compared between DOCA and sham mice at 6 weeks post surgery. N is the animal number used.

Elevated NADH Level in Cardiomyopathic Heart Tissue

[0050] We measured $[NADH]_i$ and $[NAD^+]_i$ in heart tissue of sham and DOCA mice. FIG. **1** shows that the $[NAD^+]_i$ of sham and DOCA groups were similar. On the other hand, $[NADH]_i$ was increased 2.8±0.7-fold in DOCA mice ($P<0.01$ vs. sham). According to our previous work, this amount of increase in intracellular NADH level could lead to significant decrease of I_{Na} (Reference 9). Therefore, we measured the I_{Na} of isolated myocytes of sham and DOCA mice.

Decreased I_{Na} in Cardiomyopathic Ventricular Cardiomyocytes

[0051] FIG. **2**A shows representative traces of I_{Na} measured from isolated sham and DOCA ventricular myocytes. I_{Na} of DOCA myocytes was significantly decreased. FIG. **2**B presents the averaged peak currents measured at −20 mV with a holding potential of −100 mV. I_{Na} of the cardiomyopathy group was 59±8% of the sham ($P<0.01$). The decrease in I_{Na} was not related to changes in transcription. Quantification of cardiac sodium channel (SCN5A) mRNA revealed no significant difference in transcript levels between the two groups ($P=0.95$). To investigate the Na_v 1.5 membrane expression, we marked channels present at the membrane surface with biotinylation. Western blot analysis for biotinylated Na^+ channels showed no significant difference between sham and DOCA mice (FIG. **3**: 1.19±0.16 vs. 1.24±0.17, n=3 for each group, $P=0.90$).

Restoring I_{Na} with Treatment

[0052] In our previous studies, NAD^+ and 2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (MitoTEMPO) reversed the NADH-induced decrease of I_{Na} by decreasing mitochondrial ROS production (References 9-10). Applied to isolated myocytes, NAD^+, 2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (MitoTEMPO), chelerythrine, or forskolin (500, 10, 50, or 5 μmol/L, respectively) increased I_{Na} to 113±5%, 106±8%, 129±6%, 126±3% of sham at −20 mV, respectively (FIGS. **4**A and **4**C, $P>0.05$). As shown in FIGS. **4**A and **4**B, there were minor shifts of $V_{1/2}$ values of steady state activation and inactivation, but they were not enough to affect the evaluation of the peak currents. Treatment of sham myocytes with these compounds had no effect on I_{Na}.

[0053] Treating animals with NAD^+ and 2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (MitoTEMPO) had similar effects as applying these compounds to isolated myocytes. We injected the animals twice with NAD^+ (100 mg/kg) or 2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (MitoTEMPO) (0.7 mg/kg), at 24 hours and 1 hour before the myocyte isolation. As shown in FIGS. **4**D and **4**F, NAD^+ or 2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (MitoTEMPO) completely restored the decreased I_{Na} seen in myopathic myocytes (113±5% and 106±8% of sham injected with NAD^+ or 2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl) triphenylphosphonium chloride (MitoTEMPO), respectively, at −20 mV, $P>0.05$). As shown in FIGS. **4**D and **4**E, there were also minor shifts of $V_{1/2}$ values of steady state activation and inactivation that were not enough to affect the evaluation of the peak currents.

Mitochondrial ROS are Increased in Myopathic Ventricular Myocytes

[0054] Previously, we have shown that elevated NADH increases mitochondrial ROS production causing a reduction of Na^+ current (References 9-10). To test if this mechanism of the I_{Na} reduction was similar in a clinically relevant model, MitoSOX™ Red was used to demonstrate mitochondrial ROS production in myopathic ventricular myocytes of DOCA mice. As shown in FIG. **5**A, the mitochondrial ROS level of myopathic myocytes increased -2.9±0.3-fold ($P<0.01$ vs. sham). This is similar to a four-fold increase of superoxide production observed in the aortas of DOCA mice (Reference 16).

[0055] Treatment of cells with NAD^+, 2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (MitoTEMPO), δV1-1, or forskolin (500, 10, 50, or 5 μmol/L, respectively) extracellularly led to decrease of ROS in myopathic mouse myocytes to the level similar to the sham group (1.4±0.1, 1.1±0.1, 0.9±0.1, or 0.8±0.1-fold of sham, respectively, $P>0.05$). Here we used the specific inhibitor δV1-1 of PKC instead of chelerythrine, because chelerythrine was yellow and affected the calculation

of MitoSOX™ Red fluorescence. FIG. **5**C shows representative confocal images of these measurements. Treatment of sham myocytes with NAD^+ and 2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (MitoTEMPO) had no effect on mitochondrial ROS production (data not shown). Similar results were obtained with injection of NAD^+ or 2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (MitoTEMPO) into DOCA mice (100 mg/kg or 0.7 mg/kg, respectively; FIG. **5**B). The mean fluorescent intensity of the myopathic group was increased by 1.7±0.1-fold when compared to sham ($P<0.05$). NAD^+ and 2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (MitoTEMPO) decreased the mitochondrial ROS overproduction in myopathic mouse myocytes to 1.1±0.2-fold and 1.0±0.1-fold of sham, respectively ($P>0.05$).

Discussion

[0056] Voltage-gated Na^+ channels are responsible for generating the main current for excitation propagation in the membrane of most excitable cells, such as cardiomyocytes and neurons (References 17-19). Cardiac Na^+ channel changes have been implicated in the increased risk of sudden death in heart failure (References 20-22). These changes appear to happen in the absence of significant alterations in the β-subunits of Na^+ channel, suggesting an issue involving the α-subunit (References 4 and 23). In our previous studies on the mechanism by which mutations in glycerol-3-phosphate dehydrogenase 1 like (GPD1L) protein cause reduced I_{Na} and Brugada Syndrome, we have shown that increased NADH can downregulate the cardiac Na^+ channel through PKC activation and mitochondrial ROS overproduction (References 9-10). Here, we evaluated whether the metabolic derangements occurring in cardiomyopathy could result in reductions in I_{Na} by a similar mechanism.

[0057] In this study, hypertensive mice presented with nonischemic cardiomyopathy associated with elevated intracellular NADH, increased mitochondrial ROS, and reduced I_{Na}. The reduction in I_{Na} was on the order of that seen in Brugada Syndrome, and was not the result of changes in SCN5A RNA abundance or Na_v 1.5 protein membrane expression. The reasons could be decreases of the probability of channel opening or of the single channel conductance. Further studies will be needed to differentiate these two mechanisms. The increase in mitochondrial ROS is consistent with other studies showing DOCA-salt treatment increases ROS production. Elevated oxidative stress has also been observed in the aorta of DOCA hypertension rat (References 24-25) and mice (Reference 16). Previously, we have reported this model increases cardiac oxidation (Reference 13).

[0058] Similar findings were seen in cardiomyocytes overexpressing a GPD1L mutant A280V associated with Brugada Syndrome (Reference 9). In those studies, we have demonstrated that an elevated NADH level leads to PKC activation and ROS overproduction from the mitochondrial electron transport chain (ETC) (Reference 10). In the case of the GPD1L mutant, NAD^+ and 2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (MitoTEMPO) were able to reverse the phenotype and reduce spontaneously induced arrhythmias in a mouse model of Brugada Syndrome. In this study, we found that these compounds had analogous effects to raise I_{Na} in a nonischemic cardiomyopathy model. Treating either myocytes directly or the animal, NAD^+ and 2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (MitoTEMPO) were able to reduce mitochondrial ROS production and increase I_{Na}. This suggests that while there may be other sources of oxidative stress in nonischemic cardiomyopathy, mitochondrial ROS are most important for a reduction in I_{Na}. Interestingly, 2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl) triphenylphosphonium chloride (MitoTEMPO) has also been tested in 10-day DOCA mice that showed hypertension, and reduced blood pressure has been observed (Reference 26).

[0059] We also examined the PKA activator forskolin and PKC inhibitors chelerythrine and δV1-1. They enhanced the I_{Na} and blunted the elevated mitochondrial ROS level of DOCA mice myocytes to the levels of sham mice.

[0060] This indicates that, similar to the mechanism we have found in the mutant A280V GDP1L modulation of the cardiac Na_v 1.5 (References 9-10), PKC activation participates in the signaling pathway of decreasing the I_{Na} in DOCA mice, and that PKA activation is likely involved in NAD^+ upregulation of the cardiac Na_v 1.5 of DOCA mice myocytes. The regulation of PKC on voltage-gated Na^+ channels has been studied on single channel level two decades ago (Reference 27). For the vertebrate brain type IIA Na^+ channel expressed in *Xenopus* oocytes, the open time constant was 0.26±0.05 ms, which decreased to −0.17±0.03 ms with treatment of 5 nmol/L PMA at −50 mV. Treatment of PMA also led to a reduced peak Na^+ current, reduced channel open probability, and prolonged time constants for channel activation. The restored peak I_{Na} by inhibition of PKC may be explained by the recovery of the activation process. The connection between NAD^+ and PKA has been found in many studies. Extracellular NAD^+ binds to CD38 and results in intracellular PKA activation in human granulocytes (Reference 28) and osteoblastic cells (Reference 29). PKA activation has also found to increase the release of NAD^+ in canine mesenteric artery (Reference 30). PKA phosphorylation of complex I causes an enhanced rate of NADH oxidation, reduced production of superoxide in C2C12 mouse myoblasts (Reference 31). These observations indicate a positive feedback between NAD^+ level and PKA activation. A second messenger for Ca^{2+} regulation, cADPR, is found to be downstream of PKA activation in rat cardiomyocytes (Reference 32) and human granulocytes (Reference 28). PKA activation can increase cADPR level, which leads to an increase of cytosolic Ca^{2+} concentration (References 28, 32-33). Ca^{2+} stress to isolated murine cardiac mitochondrial significantly decreases the activity of complex I and III to ~30% (Reference 34). An increase of mitochondrial Ca^{2+} concentration has been found to lead to a decrease of mitochondrial ROS level (Reference 35). In our unpublished work, we found that an antagonist of cADPR, 8-Br-cADPR, impaired forskolin's effect on recovering the I_{Na} decreased by NADH. Therefore, extracellular NAD^+ could bind to CD38, lead to PKA activation, increased cADPR level and an increase of Ca^{2+} concentration. Fluctuations of cytosolic Ca^{2+} concentration may affect the mitochondrial Ca^{2+} concentration. It is therefore possible that NAD^+ could regulate mitochondrial ROS level through this pathway.

[0061] It is well recognized that increasing severity of myopathy parallels sudden death risk (Reference 36), reduced I_{Na} increases arrhythmic risk (Reference 37), and myopathic conditions are associated with reduced I_{Na} (Ref-

erences 4-7). These studies suggest that myopathy is linked directly to reduced I_{Na} and describe a possible mechanism whereby myopathy leads to metabolic derangements resulting in increased mitochondrial ROS production causing the reduced I_{Na}. Moreover, this work suggests two possible therapies to reverse the reduced I_{Na} and possibly some of the arrhythmic risk associated with nonischemic cardiomyopathy.

[0062] Nonischemic cardiomyopathy was associated with NADH and mitochondrial ROS elevations, PKC activation and a concomitant decrease in I_{Na}. Reducing mitochondrial ROS restored I_{Na}. PKA activation likely participated in the signaling cascade of NAD^+ decreasing the mitochondrial ROS and restoring the I_{Na}. Since reduced I_{Na} and the subsequent slow conduction velocity are thought to contribute to arrhythmic risk in cardiomyopathy, NAD^+ and mitochondrial anti-oxidants may have anti-arrhythmic activity in this condition.

[0063] While this invention has been described as having preferred sequences, ranges, steps, materials, structures, components, features, and/or designs, it is understood that it is capable of further modifications, uses, and/or adaptations of the invention following in general the principle of the invention, and including such departures from the present disclosure as those come within the known or customary practice in the art to which the invention pertains, and as may be applied to the central features hereinbefore set forth, and fall within the scope of the invention and of the limits of the appended claims.

REFERENCES

[0064] The following references, including those cited in the disclosure herein, are hereby incorporated herein in their entirety by reference.

[0065] 1. Bardy G H, Lee K L, Mark D B, Poole J E, Packer D L, Boineau R, Domanski M, Troutman C, Anderson J, Johnson G, McNulty S E, Clapp-Channing N, Davidson-Ray L D, Fraulo E S, Fishbein D P, Luceri R M, Ip J H. Amiodarone or an implantable cardioverter-defibrillator for congestive heart failure. *N Engl J Med* 2005; 352(3): 225-37.

[0066] 2. Kamphuis H C M, de Leeuw J R J, Derksen R, Hauer R N W, Winnubst J A M. Implantable cardioverter defibrillator recipients: quality of life in recipients with and without ICD shock delivery. *Europace* 2003; 5(4):381-9.

[0067] 3. Thomas S A, Friedmann E, Kao C W, Inguito P, Metcalf M, Kelley F J, Gottlieb S S. Quality of life and psychological status of patients with implantable cardioverter defibrillators. *Am J Crit Care* 2006; 15(4):389-98.

[0068] 4. Valdivia C R, Chu W W, Pu J, Foell J D, Haworth R A, Wolff M R, Kamp T J, Makielski J C. Increased late sodium current in myocytes from a canine heart failure model and from failing human heart. *J Mol Cell Cardiol* 2005; 38(3):475-83.

[0069] 5. Ufret-Vincenty C A, Baro D J, Lederer W J, Rockman H A, Quinones L E, Santana L F. Role of sodium channel deglycosylation in the genesis of cardiac arrhythmias in heart failure. *J Biol Chem* 2001; 276(30):28197-203.

[0070] 6. Pu J, Boyden P A. Alterations of Na^+ currents in myocytes from epicardial border zone of the infarcted heart. A possible ionic mechanism for reduced excitability and postrepolarization refractoriness. *Circ Res* 1997; 81(1):110-9.

[0071] 7. Baba S, Dun W, Boyden P A. Can PKA activators rescue Na^+ channel function in epicardial border zone cells that survive in the infarcted canine heart? *Cardiovasc Res* 2004; 64(2):260-7.

[0072] 8. Shaw R M, Rudy Y. Ionic mechanisms of propagation in cardiac tissue: roles of the sodium and L-type calcium currents during reduced excitability and decreased gap junction coupling. *Circ Res* 1997; 81(5):727-41.

[0073] 9. Liu M, Sanyal S, Gao G, Gurung I S, Zhu X, Gaconnet G, Kerchner L J, Shang L L, Huang C L H, Grace A, London B, Dudley S C, Jr. Cardiac Na^+ current regulation by pyridine nucleotides. *Circ Res* 2009; 105(8):737-45.

[0074] 10. Liu M, Liu H, Dudley S C, Jr. Reactive oxygen species originating from mitochondria regulate the cardiac sodium channel. *Circ Res* 2010; 107(8):967-74.

[0075] 11. Aon M A, Cortassa S, Marban E, O'Rourke B. Synchronized whole cell oscillations in mitochondrial metabolism triggered by a local release of reactive oxygen species in cardiac myocytes. *J Biol Chem* 2003; 278(45): 44735-44.

[0076] 12. Di L F, Menabo R, Canton M, Barile M, Bernardi P. Opening of the mitochondrial permeability transition pore causes depletion of mitochondrial and cytosolic NAD^+ and is a causative event in the death of myocytes in postischemic reperfusion of the heart. *J Biol Chem* 2001; 276(4):2571-5.

[0077] 13. Silberman G A, Fan T-H, Liu H, Jiao Z, Xiao H D, Lovelock J D, Boulden B, Widder J, Fredd S, Bernstein K E, Wolska B, Dikalov S, Harrison D G, Dudley S C Jr. Uncoupled cardiac nitric oxide synthase mediates diastolic dysfunction. *Circulation* 2010; 121(4):519-28.

[0078] 14. O'Connor D T, Rodrigo M, Simpson P. Isolation and culture of adult mouse cardiac myocytes. *Methods Mol Biol* 2007; 357:271-96.

[0079] 15. London B, Michalec M, Mehdi H, Zhu X, Kerchner L, Sanyal S, Viswanathan P C, Pfahnl A E, Shang L L, Madhusudanan M, Baty C J, Lagana S, Aleong R, Gutmann R, Ackerman M J, McNamara D M, Weiss R, Dudley S C, Jr. Mutation in Glycerol-3-Phosphate Dehydrogenase 1-Like Gene (GPD1-L) Decreases Cardiac Na^+ Current and Causes Inherited Arrhythmias. *Circulation* 2007; 116(20):2260-8.

[0080] 16. Landmesser U, Dikalov S, Price S R, McCann L, Fukai T, Holland S, Mitch W E, Harrison D G. Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *J Clin Invest* 2003; 111(8):1201-9.

[0081] 17. Abriel H, Kass R S. Regulation of the voltage-gated cardiac sodium channel Na_v 1.5 by interacting proteins. *Trends Cardiovasc Med* 2005; 15(1):35-40.

[0082] 18. Abriel H. Cardiac sodium channel Na_v 1.5 and its associated proteins. *Arch Mal Coeur Vaiss* 2007; 100 (9):787-93.

[0083] 19. Shibata E F, Brown T L, Washburn Z W, Bai J, Revak T J, Butters C A. Autonomic regulation of voltage-gated cardiac ion channels. *J Cardiovasc Electrophysiol* 2006; 17 Suppl 1:S34-S42.

[0084] 20. Akai J, Makita N, Sakurada H, Shirai N, Ueda K, Kitabatake A, Nakazawa K, Kimura A, Hiraoka M. A novel SCN5A mutation associated with idiopathic ventricular fibrillation without typical ECG findings of Brugada syndrome. *FEBS Lett* 2000; 479(1-2):29-34.

[0085] 21. Brugada P, Brugada R, Brugada J. The Brugada syndrome. *Curr Cardiol Rep* 2000; 2(6):507-14.

[0086] 22. Makiyama T, Akao M, Tsuji K, Doi T, Ohno S, Takenaka K, Kobori A, Ninomiya T, Yoshida H, Takano M, Makita N, Yanagisawa F, Higashi Y, Takeyama Y, Kita T, Horie M. High risk for bradyarrhythmic complications in patients with Brugada syndrome caused by SCN5A gene mutations. *J Am Coll Cardiol* 2005; 46(11):2100-6.

[0087] 23. Zicha S, Maltsev V A, Nattel S, Sabbah H N, Undrovinas A I. Post-transcriptional alterations in the expression of cardiac Na^+ channel subunits in chronic heart failure. *J Mol Cell Cardiol* 2004; 37(1):91-100.

[0088] 24. Beswick R A, Zhang H, Marable D, Catravas J D, Hill W D, Webb R C. Long-term antioxidant administration attenuates mineralocorticoid hypertension and renal inflammatory response. *Hypertension* 2001; 37(2): 781-6.

[0089] 25. Beswick R A, Dorrance A M, Leite R, Webb R C. NADH/NADPH oxidase and enhanced superoxide production in the mineralocorticoid hypertensive rat. *Hypertension* 2001; 38(5):1107-11.

[0090] 26. Dikalova A E, Bikineyeva A T, Budzyn K, Nazarewicz R R, McCann L, Lewis W, Harrison D G, Dikalov S I. Therapeutic targeting of mitochondrial superoxide in hypertension. *Circ Res* 2010; 107(1):106-16.

[0091] 27. Schreibmayer W, Dascal N, Lotan I, Wallner M, Weigl L. Molecular mechanism of protein kinase C modulation of sodium channel α-subunits expressed in *Xenopus* oocytes. *FEBS Lett* 1991; 291(2):341-4.

[0092] 28. Bruzzone S, Moreschi I, Guida L, Usai C, Zocchi E, Deáflora A. Extracellular NAD^+ regulates intracellular calcium levels and induces activation of human granulocytes. *Biochem J* 2006; 393(3):697-704.

[0093] 29. Romanello M, Padoan M, Franco L, Veronesi V, Moro L, D'Andrea P. Extracellular NAD^+ induces calcium signaling and apoptosis in human osteoblastic cells. *Biochem Biophys Res Commun* 2001; 285(5):1226-31.

[0094] 30. Bobalova J, Mutafova-Yambolieva V N. Activation of the adenylyl cyclase/protein kinase A pathway facilitates neural release of -nicotinamide adenine dinucleotide in canine mesenteric artery. *Eur J Pharmacol* 2006; 536(1-2):128-32.

[0095] 31. Technikova-Dobrova Z, Sardanelli A, Speranza F, Scacco S, Signorile A, Lorusso V, Papa S. Cyclic adenosine monophosphate-dependent phosphorylation of mammalian mitochondrial proteins: enzyme and substrate characterization and functional role. *Biochemistry* 2010; 40:13941-7.

[0096] 32. Xie G H, Rah S Y, Kim S J, Nam T S, Ha K C, Chae S W, Im M J, Kim U H. ADP-ribosyl cyclase couples to cyclic AMP signaling in the cardiomyocytes. *Biochem Biophys Res Commun* 2005; 330(4):1290-8.

[0097] 33. Zhang F, Jin S, Yi F, Xia M, Dewey W L, Li P L. Local production of O_2^- by NAD(P)H oxidase in the sarcoplasmic reticulum of coronary arterial myocytes: cADPR-mediated Ca^{2+} regulation. Cell Signal 2008; 20(4):637-44.

[0098] 34. Deng N, Zhang J, Zong C, Wang Y, Lu H, Yang P, Wang W, Young G W, Wang Y, Korge P, Lotz C, Doran P, Liem D A, Apweiler R, Weiss J N, Duan H, Ping P. Phosphoproteome analysis reveals regulatory sites in major pathways of cardiac mitochondria. *Mol Cell Proteomics* 2011; 10(2):M110.000117.

[0099] 35. Kohlhaas M, Liu T, Knopp A, Zeller T, Ong M F, Bohm M, O'Rourke B, Maack C. Elevated cytosolic Na^+ increases mitochondrial formation of reactive oxygen species in failing cardiac myocytes. *Circulation* 2010; 121 (14):1606-13.

[0100] 36. Epstein A E, DiMarco J P, Ellenbogen K A, Estes N A, III, Freedman R A, Gettes L S, Gillinov A M, Gregoratos G, Hammill S C, Hayes D L, Hlatky M A, Newby L K, Page R L, Schoenfeld M H, Silka M J, Stevenson L W, Sweeney M O, Smith S C, Jr., Jacobs A K, Adams C D, Anderson J L, Buller C E, Creager M A, Ettinger S M, Faxon D P, Halperin J L, Hiratzka L F, Hunt S A, Krumholz H M, Kushner F G, Lytle B W, Nishimura R A, Ornato J P, Page R L, Riegel B, Tarkington L G, Yancy C W. ACC/AHA/HRS 2008 Guidelines for Device-Based Therapy of Cardiac Rhythm Abnormalities: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Revise the ACC/AHA/NASPE 2002 Guideline Update for Implantation of Cardiac Pacemakers and Antiarrhythmia Devices): developed in collaboration with the American Association for Thoracic Surgery and Society of Thoracic Surgeons. *Circulation* 2008; 117(21):e350-e408.

[0101] 37. Ruan Y, Liu N, Priori S G. Sodium channel mutations and arrhythmias. *Nat Rev Cardiol* 2009; 6(5): 337-48.

[0102] 38. Bogdanov K Y, Vinogradova T M, Lakatta E G. Sinoatrial nodal cell ryanodine ceceptor and Na+-Ca2+ exchanger: molecular partners in pacemaker regulation. Circ Res 2001; 88:1254-8.

[0103] 39. Lou Q, Fedorov V V, Glukhov A V, Moazami N, Fast V G, Efimov I R. Transmural heterogeneity and remodeling of ventricular excitation-contraction coupling in human heart failure/clinical perspective. Circulation 2011; 123:1881-90.

[0104] 40. Fedorov V V, Glukhov A V, Ambrosi C M, Kostecki G, Chang R, Janks D et al. Effects of KATP channel openers diazoxide and pinacidil in coronary-perfused atria and ventricles from failing and non-failing human hearts. J Mol Cell Cardiol 2011; 51:215-25.

[0105] 41. Laughner J I, Sulkin M S, Wu Z, Deng C X, Efimov I R. Three potential mechanisms for failure of high intensity focused ultrasound ablation in cardiac tissue/clinical perspective. Circ: Arrhythm Electrophysiol 2012; 5:409-16.

[0106] 42. Bayly P, KenKnight B, Rogers J, Hillsley R, Ideker R, Smith W. Estimation of conduction velocity vector fields from epicardial mapping data. IEEE Trans Biomed Eng 1998; 45:563-71.

[0107] 43. Glukhov A V, Fedorov V V, Kalish P W, Ravikumar V K, Lou Q, Janks D et al. Conduction remodeling in human end-stage nonischemic left ventricular cardiomyopathy/clinical perspective. Circulation 2012; 125:1835-47.

[0108] 44. Murphy E, Steenbergen C. Preconditioning: the mitochondrial connection. Annu Rev Physiol 2007; 69:51-67.

[0109] 45. Barth E, Stammler G, Speiser B, Schaper J. Ultrastructural quantitation of mitochondria and myofilaments in cardiac muscle from 10 different animal species including man. J Mol Cell Cardiol 1992; 24:669-81.

[0110] 46. Das D K, Maulik N. Mitochondrial function in cardiomyocytes: target for cardioprotection. Curr Opin Anaesthesiol 2005; 18:77-82.

[0111] 47. Duchen M R. Contributions of mitochondria to animal physiology: from homeostatic sensor to calcium signalling and cell death. J Physiol 1999; 516:1-17.

[0112] 48. Ide T, Tsutsui H, Kinugawa S, Utsumi H, Kang D, Hattori N et al. Mitochondrial electron transport complex I is a potential source of oxygen free radicals in the failing myocardium. Circ Res 1999; 85:357-63.

[0113] 49. Andrukhiv A, Costa A D T, West I, Garlid K D. Opening mitoKATP increases superoxide generation from complex I of the electron transport chain. Am J Physiol Heart Circ Physiol 2006; 291:H2067-H2074.

[0114] 50. Eaton P. Protein thiol oxidation in health and disease: techniques for measuring disulfides and related modifications in complex protein mixtures. Free Radic Biol Med 2006; 40:1889-99.

[0115] 51. Winterbourn C C. Reconciling the chemistry and biology of reactive oxygen species. Nat Chem Biol 2008; 4:278-86.

[0116] 52. Santos C X C, Anilkumar N, Zhang M, Brewer A C, Shah A M. Redox signaling in cardiac myocytes. Free Radic Biol Med 2011; 50:777-93.

[0117] 53. Lovelock J D, Monasky M M, Jeong E M, Lardin H A, Liu H, Patel B G et al. Ranolazine improves cardiac diastolic dysfunction through modulation of myofilament calcium sensitivity. Circ Res 2012; 110:841-50.

[0118] 54. Zhou J, Shin H G, Yi J, Shen W, Williams C P, Murray K T. Phosphorylation and putative ER retention signals are required for protein kinase A-mediated potentiation of cardiac sodium current. Circ Res 2002; 91:540-6.

[0119] 55. Tateyama M, Kurokawa J, Terrenoire C, Rivolta I, Kass R S. Stimulation of protein kinase C inhibits bursting in disease-linked mutant human cardiac sodium channels. Circulation 2003; 107:3216-22.

What is claimed is:

1. A method for reducing arrhythmic risk associated with cardiomyopathy, comprising the step of administering a composition containing NAD^+ to an individual in need thereof.

2. The method of claim **1**, wherein the amount of NAD^+ is effective to reduce arrhythmic risk.

3. The method of claim **1**, wherein the amount of NAD^+ is effective to achieve a NAD^+ blood concentration of about 100-500 μM.

4. The method of claim **1**, wherein the individual is suffering from arrhythmia.

5. The method of claim **1**, wherein the administering step comprises intravenous administration of the composition.

6. The method of claim **1**, wherein the composition comprises at least one member selected from the group consisting of a powder, a tablet, a capsule, a solution, a suspension, and an injectable formulation.

7. The method of claim **1**, wherein the NAD^+ is about 0.1 to about 90% by weight of the composition.

8. The method of claim **1**, wherein the composition is a solution for intravenous administration.

9. A method for restoring the cardiac sodium current to a normal level in an individual with cardiomyopathy, comprising the step of administering a composition containing NAD^+ to an individual in need thereof.

10. The method of claim **9**, wherein the amount of NAD^+ is effective to reduce arrhythmic risk.

11. The method of claim **9**, wherein the amount of NAD^+ is effective to achieve a NAD^+ blood concentration of about 100-500 μM.

12. The method of claim **9**, wherein the individual is suffering from arrhythmia.

13. The method of claim **9**, wherein the administering step comprises intravenous administration of the composition.

14. The method of claim **9**, wherein the composition comprises at least one member selected from the group consisting of a powder, a tablet, a capsule, a solution, a suspension, and an injectable formulation.

15. The method of claim **9**, wherein the NAD^+ is about 0.1 to about 90% by weight of the composition.

16. The method of claim **9**, wherein the composition is a solution for intravenous administration.

17. A method for reducing arrhythmic risk in an individual with cardiomyopathy and a cardiac ejection fraction of less than 50%, comprising the step of administering a composition containing NAD^+ to an individual in need thereof.

18. The method of claim **17**, wherein the amount of NAD^+ is effective to reduce arrhythmic risk.

19. The method of claim **17**, wherein the amount of NAD^+ is effective to achieve a NAD^+ blood concentration of about 100-500 μM.

20. The method of claim **17**, wherein the individual is suffering from arrhythmia.

21. The method of claim **17**, wherein the administering step comprises intravenous administration of the composition.

22. The method of claim **17**, wherein the composition comprises at least one member selected from the group consisting of a powder, a tablet, a capsule, a solution, a suspension, and an injectable formulation.

23. The method of claim **17**, wherein the NAD^+ is about 0.1 to about 90% by weight of the composition.

24. The method of claim **17**, wherein the composition is a solution for intravenous administration.

25. A method for reducing arrhythmic risk associated with cardiomyopathy, comprising the step of administering a mitochondrial targeted antioxidant to an individual in need thereof.

26. The method of claim **25**, wherein the amount of antioxidant is effective to reduce arrhythmic risk.

27. The method of claim **25**, wherein the antioxidant prevents or lowers reduction in sodium channel current (I_{Na}) by reducing or suppressing mitochondrial ROS production.

28. The method of claim **25**, wherein the antioxidant is administered orally or intravenously.

29. The method of claim **25**, wherein the individual is suffering from arrhythmia.

30. The method of claim **25**, wherein the antioxidant comprises at least one member selected from the group consisting of a powder, a tablet, a capsule, a solution, a suspension, and an injectable formulation.

31. The method of claim **25**, wherein the antioxidant comprises 2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (MitoTEMPO).

32. A method for restoring the cardiac sodium current to a normal level in an individual with cardiomyopathy, comprising the step of administering a mitochondrial targeted antioxidant to an individual in need thereof.

33. The method of claim **32**, wherein the amount of antioxidant is effective to reduce arrhythmic risk.

34. The method of claim **32**, wherein the antioxidant prevents or lowers reduction in sodium channel current (I_{Na}) by reducing or suppressing mitochondrial ROS production.

35. The method of claim **32**, wherein the antioxidant is administered orally or intravenously.

36. The method of claim **32**, wherein the individual is suffering from arrhythmia.

37. The method of claim **32**, wherein the antioxidant comprises at least one member selected from the group consisting of a powder, a tablet, a capsule, a solution, a suspension, and an injectable formulation.

38. The method of claim **32**, wherein the antioxidant comprises 2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (MitoTEMPO).

39. A method for reducing arrhythmic risk in an individual with cardiomyopathy and a cardiac ejection fraction of less than 50%, comprising the step of administering a mitochondrial targeted antioxidant to an individual in need thereof.

40. The method of claim **39**, wherein the amount of antioxidant is effective to reduce arrhythmic risk.

41. The method of claim **39**, wherein the antioxidant prevents or lowers reduction in sodium channel current (I_{Na}) by reducing or suppressing mitochondrial ROS production.

42. The method of claim **39**, wherein the antioxidant is administered orally or intravenously.

43. The method of claim **39**, wherein the individual is suffering from arrhythmia.

44. The method of claim **39**, wherein the antioxidant comprises at least one member selected from the group consisting of a powder, a tablet, a capsule, a solution, a suspension, and an injectable formulation.

45. The method of claim **39**, wherein the antioxidant comprises 2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (MitoTEMPO).

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