Modulation of the Cardiac Sodium Channel Nav1.5 Peak and Late Currents by NAD⁺ Precursors

Daniel S. Matasic^{a,b,c}, Jin-Young Yoon^{a,b}, Jared M. McLendon^{a,b}, Haider Mehdi^{a,b}, Mark S. Schmidt^{b,d}, Alexander M. Greiner^{a,b}, Pravda Quinones^{a,b}, Gina M. Morgan^{a,b}, Ryan L. Boudreau^{a,b}, Kaikobad Irani^{a,b}, Charles Brenner^{b,d}, and Barry London^{a,b,c*}

*corresponding author

^aDivision of Cardiovascular Medicine, Department of Internal Medicine, University of Iowa Carver College of Medicine, Iowa City, IA

^bAbboud Cardiovascular Research Center, University of Iowa Carver College of Medicine, Iowa City, IA

^cDepartment of Molecular Physiology and Biophysics, University of Iowa Carver College of Medicine, Iowa City, IA

^dDepartment of Biochemistry, University of Iowa Carver College of Medicine, Iowa City, IA

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Address correspondence to:

Barry London, MD PhD
Professor and Director, Division of Cardiovascular Medicine
University of Iowa
285 Newton Road, 2283 CBRB
Iowa City, Iowa 52242
barry-london@uiowa.edu

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ABSTRACT

Rationale: The cardiac sodium channel Nav1.5, encoded by *SCN5A*, produces the rapidly inactivating depolarizing current I_{Na} that is responsible for the initiation and propagation of the cardiac action potential. Acquired and inherited dysfunction of Nav1.5 results in either decreased peak I_{Na} or increased residual late I_{Na} ($I_{Na,L}$), leading to tachy/bradyarrhythmias and sudden cardiac death. Previous studies have shown that increased cellular NAD^+ and $NAD^+/NADH$ ratio increase I_{Na} through suppression of mitochondrial reactive oxygen species and PKC-mediated Nav1.5 phosphorylation. In addition, NAD^+ -dependent deacetylation of Nav1.5 at K1479 by Sirtuin 1 increases Nav1.5 membrane trafficking and I_{Na} . The role of NAD^+ precursors in modulating I_{Na} remains unknown.

Objective: To determine whether and by which mechanisms the NAD^+ precursors nicotinamide riboside (NR) and nicotinamide (NAM) affect peak I_{Na} and $I_{Na,L}$ *in vitro* and cardiac electrophysiology *in vivo*.

Methods and Results: The effects of NAD^+ precursors on the NAD^+ metabolome and electrophysiology were studied using HEK293 cells expressing wild-type and mutant Nav1.5, rat neonatal cardiomyocytes (RNCMs), and mice. NR increased I_{Na} in HEK293 cells expressing Nav1.5 (500 μ M: $51 \pm 18\%$, $p=0.02$, 5 mM: $59 \pm 22\%$, $p=0.03$) and RNCMs (500 μ M: $60 \pm 26\%$, $p=0.02$, 5 mM: $75 \pm 39\%$, $p=0.03$) while reducing $I_{Na,L}$ at the higher concentration (RNCMs, 5 mM: $-45 \pm 11\%$, $p=0.04$). NR (5 mM) decreased Nav1.5 K1479 acetylation but increased I_{Na} in HEK293 cells expressing a mutant form of Nav1.5 with disruption of the acetylation site (Nav1.5-K1479A). Disruption of the PKC phosphorylation site abolished the effect of NR on I_{Na} . Furthermore, NAM (5 mM) had no effect on I_{Na} in RNCMs or in HEK293 cells expressing wild-type Nav1.5, but increased I_{Na} in HEK293 cells expressing Nav1.5-

K1479A. Dietary supplementation with NR for 10-12 weeks decreased QTc in C57BL/6J mice (0.35% NR: $-4.9 \pm 2.0\%$, $p=0.26$; 1.0% NR: $-9.5 \pm 2.8\%$, $p=0.01$).

Conclusions: NAD⁺ precursors differentially regulate Nav1.5 via multiple mechanisms. NR increases I_{Na}, decreases I_{Na,L}, and warrants further investigation as a potential therapy for arrhythmic disorders caused by Nav1.5 deficiency and/or dysfunction.

KEYWORDS:

Cardiac Sodium Channel (Nav1.5, SCN5A)

Arrhythmia

Metabolic Regulation, NAD⁺/NADH

Nicotinamide Riboside, Nicotinamide

Sirtuins, Acetylation

1. INTRODUCTION

The main cardiac sodium channel Nav1.5 generates a rapid inward depolarizing Na⁺ current (I_{Na}) that initiates and allows propagation of the cardiac action potential throughout the heart.¹ Loss-of-function mutations in *SCN5A*, the gene encoding the pore-forming alpha subunit of Nav1.5, result in: (1) a reduction of I_{Na}, (2) a decrease in conduction velocity, and (3) an increased risk for tachyarrhythmias, bradyarrhythmias, and sudden cardiac death.²⁻⁵ Inherited deficiency in Nav1.5 can manifest as several conditions including Brugada Syndrome, sick sinus syndrome, and progressive cardiac conduction defects, whereas gain-of-function mutations in *SCN5A* that limit voltage- and time-dependent inactivation increase late Na⁺ current (I_{Na,L}) and result in Long QT syndrome type 3 (LQT3).⁶⁻⁹ Moreover, acquired dysfunction of Nav1.5 in conditions such as cardiomyopathies and heart failure can decrease I_{Na} and increase I_{Na,L}, contributing to fatal arrhythmias.¹⁰ Given the importance of Nav1.5 in modulating arrhythmic risk, efforts are underway to modulate Nav1.5 expression and/or activity as a potential therapeutic avenue for arrhythmogenesis in inherited and acquired conditions.

The cellular redox coenzyme nicotinamide adenine dinucleotide (NAD⁺) has been established as an important regulator of cardiac physiology and pathophysiology.¹¹ Previous studies have shown that NAD⁺ supplementation can normalize the NAD⁺/NADH redox ratio and increase I_{Na}.¹² Additionally, NAD⁺ supplementation and increased NAD⁺/NADH ratio have been shown to alter protein kinase A (PKA) and protein kinase C (PKC) dependent Nav1.5 phosphorylation, thereby altering Nav1.5 surface expression and/or channel conductance.¹²⁻¹⁴ More recently, we showed that the NAD⁺-dependent deacetylase Sirtuin-1 (SIRT1) increases Nav1.5 surface

expression and I_{Na} by deacetylating a lysine residue (K1479) within the Nav1.5 III-IV intracellular linking domain *in vitro* and in animal models.^{15, 16}

Given that NAD^+ has poor bioavailability, NAD^+ precursors are emerging as a therapeutic strategy for the prevention of cardiovascular disease.¹¹ Nicotinamide Riboside (NR), a bioavailable and well-tolerated oral NAD^+ precursor, has emerged as a leading NAD^+ supplement in humans. Several ongoing clinical trials examining the effects of NR supplementation on cardiovascular diseases including heart failure are currently underway.¹⁷⁻²⁰ However, the effects of NAD^+ precursor supplementation on Nav1.5 function and cardiac electrophysiology are relatively unexplored.

In this report, we demonstrate that the NAD^+ precursor NR alters the NAD^+ metabolome, increasing NAD^+ content, Nav1.5 deacetylation, and peak I_{Na} while reducing $I_{Na,L}$. In addition, nicotinamide (NAM), an alternative NAD^+ precursor known to inhibit sirtuin activity at high doses, does not increase I_{Na} at high dose in either HEK293 cells or rat neonatal cardiomyocytes (RNCMs). We demonstrate that the effect of NR is mediated by the PKC-phosphorylation S1503 site on Nav1.5 and that the inhibitory effect of NAM is mediated by the K1479 acetylation site. Furthermore, dietary supplementation of 1% NR in wild-type mice resulted in a reduction in QTc after 10-12 weeks, consistent with the reduction in $I_{Na,L}$. Together, this work lays a foundation for further investigation of the NAD^+ precursor NR as a potential therapeutic agent for the prevention of arrhythmias in cardiac diseases associated with Nav1.5 deficiency and/or dysfunction.

2. METHODS

All animal studies were approved by the Institutional Animal Care and Use Committees (IACUC) at the University of Iowa.

2.1 Cell Culturing of Non-Myocytes and Myocytes

HEK293 cells and HEK293 cells stably-expressing Nav1.5 were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and Dr. Samuel Dudley (University of Minnesota), respectively, and cultured in 10%-fetal bovine serum and 1% penicillin/streptomycin supplemented Dulbecco's Modified Eagle's Medium (DMEM). RNCMs were isolated from 1-3 day old Sprague-Dawley rat pups using a neonatal cardiomyocyte isolation system (Worthington Biochemical Co, Lakewood, NJ) and cultured in 5%-horse serum supplemented DMEM/F-12 equivolumetric mixture.

2.2 Reagents

Transient expression of Nav1.5 constructs in HEK293 cells was achieved utilizing Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Mutant Nav1.5-K1479A and Nav1.5-K1479A/S1503A were generated by site-directed mutagenesis using the Quik-Change II XL kit (Agilent Technologies, Santa Clara, CA). The following NAD⁺ metabolites were utilized: nicotinamide riboside (NR, Niagen, Irvine, CA), nicotinamide (NAM, Sigma-Aldrich, St. Louis, MO), and 1-methylnicotinamide (meNAM, Sigma-Aldrich, St. Louis, MO). In experiments using transient transfection of Nav1.5, treatment of NAD⁺ metabolites was initiated 12 hours post-transfection, continued for 48 hours, and cells were subjected to whole-cell patch clamp after

plating onto poly-D-lysine-coated coverslips. In experiments using RNCMs, treatment with NAD^+ metabolites was continued for 24 hours post-plating onto laminin-coated coverslips.

2.3 Whole-Cell-Patch Clamp of Na^+ Currents (I_{Na})

All whole-cell recordings were obtained utilizing the Axon Axopatch 200B amplifier and Digidata 1440B data acquisition system (Molecular Devices, San Jose, CA), as previously described.¹⁵ Treatment with NAD^+ metabolites was continued throughout experimentation with extracellular solutions containing the respective concentration of the respective metabolite. Cell capacitance was recorded directly from the amplifier after adjusting for the transient post-membrane rupture.

For HEK293 cells, patch pipettes of 2-3 $\text{M}\Omega$ were filled with an internal solution containing: 110 mM CsF, 20 mM CsCl, 10 mM NaF, 10 mM EGTA, 10 mM HEPES, with the pH adjusted to 7.35 with CsOH. The extracellular solution contained: 25 mM NaCl, 128 mM NMDG, 4.5 mM KCl, 10 mM HEPES, 1 mM MgCl_2 , 1.5 mM CaCl_2 , 5 mM glucose, and pH adjusted to 7.35 using HCl. To test the steady-state activation of I_{Na} in HEK293 cells, a 500-ms prepulse to -120 mV was initiated to recover any channels from inactivation and then cells were subjected to a 20 or 200-ms test pulse between -90 mV and +40 mV in increments of 5 mV. The peak current density was assessed at the test potential of largest inward current. To test the steady-state inactivation, a two-pulse protocol was employed with 500-ms conditioning pulses varying from -140 mV to -30 mV, followed by a 20-ms test pulse at -20 mV.

For RNCMs, patch pipettes of 2-3 M Ω were filled with an internal solution containing: 10 mM NaCl, 90 mM aspartic acid, 70 mM CsOH, 10 mM EGTA, 20 mM CsCl, 10 mM HEPES, and pH adjusted to 7.35 using CsOH. The extracellular solution contained: 25 mM NaCl, 120 mM CsCl, 4.5 mM KCl, 10 mM HEPES, 2 mM MgCl₂, 0.5 mM CaCl₂, and pH adjusted to 7.35 using CsCl. To test the steady-state activation of I_{Na} in RNCMs, a 200-ms prepulse to -120 mV was used to eliminate any inactivated channels and then cells were subjected to a 200-ms test pulse between -80 mV and +15 mV in increments of 5 mV. To test the steady-state inactivation, a two-pulse protocol was employed with 1-s conditioning pulses varying from -150 mV to +30 mV followed by a 200-ms test pulse at -20 mV. $I_{Na,L}$ was calculated by averaging the current between 50-ms and 150-ms of the 200-ms depolarization at three test-potentials: 20 mV, 25 mV, 30 mV). To verify the measurements of $I_{Na,L}$ in RNCMs, a 143 mM NaCl extracellular solution was used and RNCMs were subjected to a continuous protocol of a 200-ms prepulse to -120 mV followed by a 200-ms depolarizing test-pulse to -20 mV with a 5-s interpulse duration at a holding potential of -80 mV before and after application of 10 μ M tetrodotoxin (TTX). TTX-sensitive $I_{Na,L}$ was calculated by the difference of measured late current before and after TTX.

pClamp software (version 10.4) was utilized for data analysis. To calculate I_{Na} , peak current was normalized to the membrane capacitance. Steady-state activation and inactivation curves were generated and fitted to the Boltzmann equation, whereupon half-maximal potential ($V_{1/2}$) and slope factor (k) were derived.

2.4 Whole-Cell-Patch Clamp of K^+ and Ca^{2+} Currents

Conventional whole-cell patch clamp techniques were used to record K^+ and Ca^{2+} currents from RNCM. Total potassium current was evoked during 4-s depolarizing voltage steps to potentials between -110 and 50 mV from a holding potential of -80 mV in 10 mV increments. Each trial was preceded by a short (20-ms) depolarization to -20 mV to eliminate contamination of Na^+ currents. $I_{K,total}$ was defined as the currents taken 20-ms into depolarization, and $I_{K,sustained}$ as the currents taken just prior to repolarization. I_{to} was determined by the difference between $I_{K,total}$ and $I_{K,sustained}$. Rapidly activating, slowly inactivating outward current (I_{Kur}) was obtained by subtraction of currents evoked by depolarizing voltage steps (-110 to 50 mV in 10 mV increments) from a holding potential of -40 mV and -80 mV followed by a 100-ms -40 mV inactivating prepulse. Ca^{2+} current (I_{Ca}) was elicited by holding at -80 mV and stepping between -70 and 70 mV (10 mV steps) for 500-ms after a prepulse to -40 mV for 1-s to inactivate Na^+ currents.

The internal pipette solution for I_K voltage clamp studies was: 140 mM KCl, 4 mM Mg-ATP, 1 mM $MgCl_2$, 5 mM EGTA, 10 mM HEPES; the extracellular solution contained: 143 mM NaCl, 4.5 mM KCl, 10 mM HEPES, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.1 mM $CdCl_2$. The pH was adjusted to 7.4 with NaOH. The internal pipette solution for I_{Ca} recording was: 110 mM CsCl, 20 mM TEACl, 5 mM Mg-ATP, 2 mM Na_2 -ATP, 5 mM Na Creatine Phosphate, 5 mM HEPES, 3 mM EGTA, and pH was adjusted to 7.2 with TRIS. The extracellular solution was: 130 mM NMDG, 20 mM Tetraethylammonium chloride (TEACl), 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, 10 mM Glucose, and the pH was adjusted to 7.4 with HCl. Cells were dialyzed for 5 min prior to initiating experimental protocols.

2.5 Quantification of the NAD⁺/NADH Ratio and the NAD⁺ Metabolome

NAD⁺, NADH, and NAD⁺/NADH ratios was quantified in cellular extracts using the NAD/NADH-GloTM Assay Kit (Promega Co, Madison, WI). Direct measurements of NAD⁺ and other metabolites in the NAD⁺ metabolome were performed on cell extracts using Liquid Chromatography-Mass Spectrometry (LC-MS), as previously described.²¹ Measurements below the quantitation limit were set to the minimum value of quantitation.

2.6 Western Blotting

Conventional techniques were utilized as previously described.¹⁵ To assess the acetylation of K1479, a custom-designed anti-acetyl K1479 Nav1.5 antibody was utilized, as previously described and used.¹⁵ Total Nav1.5 expression in whole cell lysates (Alomone ASC-005) was normalized to GAPDH (Trevigen 2275-PC). To measure the membrane expression of Nav1.5, the membrane-enriched protein fraction was harvested with the Compartmentalization Protein Extraction Kit (Millipore, Burlington, MA).

2.7 Assessment of Nav1.5 Surface Expression by Microscopy

HEK293 cells that stably express Nav1.5 with an extracellular FLAG epitope in the extracellular region of Domain I were seeded on gelatin coated glass bottom 24-well plates (Cellvis, Sunnyvale, CA). These FLAG-Nav1.5 HEK293 cells were treated for 48 hours with 5 mM NR (replenished media at treatment at 24 hours) and subsequently fixed for 15 minutes with 4% paraformaldehyde (diluted from 16%, Electron Microscopy Sciences, Hatfield, PA). Without cell permeabilization, cells were incubated in blocking buffer (PBS with 2% BSA 5% goat serum)

for 1 hour, and then incubated overnight with Anti-FLAG M2 Antibody (Sigma Aldrich, St. Louis MO), diluted 1:1000 in blocking buffer, followed by a one hour incubation in Alexa-568 conjugated anti-mouse antibody (Abcam, Cambridge UK) diluted 1:1000 in blocking buffer. Surface Expression of FLAG-Nav1.5 was assessed by confocal microscopy using a Zeiss LSM510 microscope with a 63x objective (Plan Apochromat 63x/1.4 oil DIC) and Zeiss ZEN software.

2.8 Dietary Supplementation of Nicotinamide Riboside in Mice

4-5-month-old C57BL/6J mice of both sexes were randomly placed on a control diet (Teklad 2920X, Envigo) or a diet supplemented with NR at either 0.35% or 1% (3.5 g or 10 g of NR, respectively, in 1 kg of Teklad 2920X, Envigo) for 10-12 weeks. Food was prepared fresh every 3-4 days and mice were weighed to assess for any changes in body weight throughout the experiment.

2.9 Electrocardiography and Echocardiography in Mice

Electrocardiograms (EKGs) were performed at baseline, 4-6 weeks of diet, and 10-12 weeks of diet using the iWork IX/100B EKG recording system. The mice were anesthetized with 1-2% isoflurane, and high resolution multi-lead EKGs were obtained by placing bipolar electrodes subcutaneously in positions corresponding to human leads: I (right to left arm), II (right arm to left leg), III (left arm to left leg), and modified chest V (back to left sternal border), as previously described.²² Data was collected, digitized, and analyzed using LabScribe 2.0 software (iWorx, Dover, NH). An investigator blinded by treatment group analyzed R-R interval, PR interval,

QRS duration, and QT interval for leads I, II, III, and V. QTc was calculated as previously described.²³ Maximum values among leads for PR, QRS, and QTc are reported.

Transthoracic echocardiography was performed to assess cardiac size and function at baseline and 10-12 weeks of diet (Vevo 2700 VisualSonics System). Mice were anesthetized with midazolam; measurements included heart rate, end diastolic volume, end systolic volume, and ejection fraction as previously described.²⁴

2.10 Statistical Analysis

All statistical analysis was performed with GraphPad Prism. Results are represented as the mean $\pm$ standard error of the mean and considered statistically significant if p-values were ≤ 0.05 . All analyses were performed blinded to treatment or construct, as appropriate. Significance of difference between two groups was evaluated using independent sample t-tests. In addition, two-way ANOVAs were used to evaluate differences between groups over time.

3. RESULTS

3.1 Nicotinamide Riboside Increases I_{Na} in HEK293 Cells Expressing Nav1.5 and in RNCMs

Previous studies showed that extracellular application of NAD^+ reversed the downregulation of I_{Na} that resulted from increased NADH levels in HEK293 cells expressing Nav1.5.¹² We tested whether NR (Figure 1A), a bioavailable precursor of NAD^+ , can influence I_{Na} . NR (500 μ M) supplementation of HEK293 cells constitutively expressing Nav1.5 increased NAD^+ and NADH levels without changing the $NAD^+/NADH$ ratio (Figure 1B), and increased I_{Na} ($51 \pm 18\%$, $p=0.02$; Figure 1C-E, Table S1) with only minor changes to Nav1.5 gating properties (4 mV hyperpolarizing shift in the steady-state activation half-potential; Figures 1F, S1). Similarly, 5 mM NR significantly increased I_{Na} in HEK293 cells transiently-transfected with the wild-type Nav1.5 channel ($59 \pm 22\%$, $p=0.03$, Figure 1G-H, Table S1) with no effects on their gating properties (Figures 1I, S1, Table S5). Surface expression of the channel was not increased in response to NR (Figures S2, S3).

Comparable results were observed in RNCMs (Figure 2). Representative whole-cell traces and I-V curves illustrated the stimulatory effects of NR on I_{Na} (500 μ M NR: $60 \pm 26\%$, $p=0.02$; 5 mM NR: $74 \pm 39\%$, $p=0.03$; Figure 2A-C, Table S2). Changes in gating properties including steady-state activation and inactivation were observed after treatment with 5 mM NR but not 500 μ M NR, with a depolarizing shift of steady-state activation and inactivation (Figures 2D, S4, Table S5). Interestingly, 5 mM NR reduced $I_{Na,L}$ ($-45 \pm 11\%$, $p=0.04$, measured at -25 mV; Figure 2E-F). The absolute late current densities, measured at -25 mV, for 5 mM NR relative to control (-1.88 ± 0.23 pA/pF vs. -3.32 ± 0.26 pA/pF, $p=0.002$, Figure S5) also decreased. To verify that the

observed changes in $I_{Na,L}$ were not attributed to contaminated leak currents, $I_{Na,L}$ was also determined by measuring the TTX-sensitive current. 5 mM NR significantly decreased the TTX-sensitive $I_{Na,L}$ compared to control in RNCMs (-1.98 ± 0.48 pA/pF vs. -4.43 ± 1.24 pA/pF, $p=0.04$, Figure 2G-H). NR increased NAD^+ and NADH content in RNCMs, similar to the findings in HEK293 cells; however, NR also significantly increased the $NAD^+/NADH$ ratio in RNCMs (Figure 2I).

3.2 Nicotinamide Riboside Drives Deacetylation of Nav1.5

Our group recently showed that the NAD^+ -consuming enzyme Sirtuin 1 regulates Nav1.5 trafficking by deacetylation of a lysine residue at the 1479 position within the III-IV intracellular linker domain.¹⁵ NR supplementation of HEK293 cells transiently transfected with Nav1.5 decreased the acetylation of K1479 only at the higher 5 mM dose, with no effect on total Nav1.5 mRNA or protein expression (Figures 3, S6).

3.3 Nicotinamide Riboside does not alter K^+ currents or Ca^{2+} currents

To assess whether NR has an influence on other currents regulating the cardiac action potential, K^+ currents and Ca^{2+} currents were assessed by whole-cell patch clamp in RNCMs after a 24 hour treatment with 5 mM NR. NR had no significant effect on K^+ currents including total I_K , sustained I_K , I_{to} , and I_{Kur} . NR had no effect on Ca^{2+} current density and inactivation kinetics in RNCMs as assessed by whole cell patch clamp (Figure S7).

3.4 Nicotinamide has Differential Effects on I_{Na} between HEK293 Cells and RNCMs

NAM (Figure 4A), an alternate NAD^+ precursor to NR known to inhibit sirtuins at high dose, had no significant effect on I_{Na} in HEK293 cells transiently-transfected with Nav1.5 (500 μM : $-19 \pm 26\%$, $p=0.56$; 5 mM: $+21 \pm 26\%$, $p=0.49$; Figure 4B-C, Table S3). No significant changes were observed in steady-state activation and inactivation after NAM administration (Figure S8). Interestingly, NAM stimulated I_{Na} in RNCMs at a lower dose (500 μM : $84 \pm 33\%$, $p=0.003$) but had no significant effect on I_{Na} at a higher dose (5 mM: $-11 \pm 24\%$, $p=\text{NS}$; Figure 4D-E, Table S3). Gating properties were unchanged (Figures 4F, S9, Table S5). Unlike NR, NAM had no effect of $I_{\text{Na,L}}$ (Figure 4G). Measurement of NAD^+ and NADH showed that NAM increased both NAD^+ and NADH content in a dose-dependent manner with a significant increase in the overall NAD^+/NADH ratio (500 μM : $7.5 \pm 2.0\%$, $p=0.007$, 5 mM: $23.8 \pm 3.9\%$, $p<0.0001$; Figure 4H).

3.5 1-methyl-Nicotinamide, an inert Nicotinamide Metabolite, has no effect on I_{Na}

1-methyl-Nicotinamide (meNAM), an inert byproduct of NAM degradation and not an NAD^+ precursor, had no significant effect on I_{Na} (500 μM : $-7 \pm 14\%$, $p=\text{NS}$; 5 mM: $-5 \pm 9.9\%$, $p=\text{NS}$) channel gating properties, or $I_{\text{Na,L}}$ in RNCMs (Figure S10A-G). MeNAM tended to increase NAD^+ and decrease NADH content in a dose-dependent manner, leading to a significant increase in NAD^+/NADH ratio (Figure S10H).

3.6 NAD^+ Metabolome Following NAD^+ Metabolite Supplementation in RNCMs

NAD^+ metabolite supplementation in cardiomyocytes has been relatively unexplored. The effect of supplementation with NR, NAM or meNAM on the RNCM NAD^+ metabolome was assessed by LC-MS (Figure 5). Both NR and NAM caused dose-dependent increases in NAD^+ whereas the inert meNAM had no effect on NAD^+ content (Figure 5A). NR was superior to NAM in

increasing NAD^+ and nicotinamide adenine dinucleotide phosphate (NADP^+) at equimolar concentrations (Figure 5A,B). However, NAM was superior in increasing nicotinic acid adenine dinucleotide (NAAD, Figure 5C). NR markedly depressed levels of ADP-ribose (ADPR), a substrate for another class of NAD^+ -consuming enzymes, Poly-ADP-Ribose Polymerases (PARPs, Figure 5I). NAM, an inhibitor of PARPs, had no significant effect on ADPR levels.

3.7 Disruption of the $\text{Nav}1.5$ -K1479 Acetylation Site and $\text{Nav}1.5$ -S1503 Phosphorylation sites Modulates the Effects of NAD^+ Precursors on I_{Na}

SIRT1-dependent deacetylation of $\text{Nav}1.5$ in the intracellular Domain III-IV linker at K1479 increased I_{Na} by enhancing membrane trafficking, and disruption of the acetylation by site-directed mutagenesis ($\text{Nav}1.5$ -K1479A) eliminated the effect of SIRT1 on I_{Na} in HEK293 cells.¹⁵ To determine the role of acetylation following treatment with NAD^+ precursors, HEK293 cells transiently transfected with wild-type $\text{Nav}1.5$ or $\text{Nav}1.5$ -K1479A channels were treated with NR or NAM. No differences in the magnitude and gating properties of I_{Na} were observed between the wild-type and K1479A $\text{Nav}1.5$ channels under basal conditions (Figure S11). Similar to its effect on wild-type channels (Figure 1G-H), NR increased I_{Na} in HEK293 cells expressing $\text{Nav}1.5$ -K1479A (5 mM: $75 \pm 29\%$, $p=0.02$; Figure 6A,B) and significantly shifted steady-state activation and inactivation to more hyperpolarized potentials (Figures 6C, S12, Table S5). However, NR had no effect on I_{Na} in HEK293 cells expressing $\text{Nav}1.5$ -K1479A/S1503A (5 mM: $16 \pm 12\%$, $p=0.38$; Figure 6D,E) while maintaining the hyperpolarizing shift in steady-state activation and inactivation (Figures 6F, S12, Table S5). Therefore, disruption of the PKC site abolished the effect of NR on increasing I_{Na} .

Administration of NAM to HEK293 cells expressing Nav1.5-K1479A increased I_{Na} (500 μ M: $81 \pm 47\%$, $p=0.10$; 5 mM: $66 \pm 25\%$, $p=0.04$; Figure 6G-I), unlike our prior finding that NAM had no effect on HEK293 cells expressing wild-type Nav1.5 (Figure 4). NAM had no effect on steady-state activation or inactivation (Figure S8). Thus, elimination of the SIRT1-deacetylation site allowed NAM to increase I_{Na} .

Together these results suggest that both SIRT1-dependent and SIRT1-independent mechanism are modulating Nav1.5 in response to NAD^+ metabolites that increase NAD^+ .

3.8 Dietary Supplementation of Nicotinamide Riboside Decreases QTc in Wild-type Mice

To assess for changes in cardiac electrophysiology with NAD^+ supplementation *in vivo*, C57BL/6J mice were either placed on a diet with (0.35% or 1.0% by weight) or without (control) supplemental NR. Over the time course of dietary supplementation, no differences in body weight were observed demonstrating that the NR diets are tolerated and did not alter growth (Figure S13). Electrophysiological properties were assessed by high-resolution EKG (Figure 7A-B). No differences in PR and QRS intervals were observed between groups at different timepoints (Figure 7C-D, Table S6). However, mice supplemented with NR demonstrated a decrease in QTc (0.35% NR: $-4.9 \pm 2.0\%$, $p=0.22$, 1.0% NR: $-9.5 \pm 2.8\%$, $p=0.01$) after 10-12 weeks compared to baseline, with no change in mice on a control diet ($+1.8 \pm 2.3\%$, $p=0.99$, Figure 7A,B,E, Table S6). NR supplementation had no effect on cardiac function as assessed by echocardiography (Table S7).

4. DISCUSSION

Our group and others have explored the molecular basis for the regulation of the cardiac sodium channel Nav1.5 by NAD⁺, NADH, and the NAD⁺/NADH ratio. Previous studies demonstrated that exogenous application of 100 μM NAD⁺ on Langendorff-perfused hearts from *Scn5a* haplo-insufficient mice was protective against programmed electrical stimulation induced polymorphic ventricular tachycardia.¹² In addition, exogenous *in vitro* application and *in vivo* intraperitoneal injection of NAD⁺ rescued the decrease in peak I_{Na} in myocytes from mice with a non-ischemic cardiomyopathy (with hypertension from unilateral nephrectomy, deoxycorticosterone acetate pellet implantation, and salt water substitution).²⁵ Furthermore, application of NAD⁺ on failing human heart wedge preparations improved conduction velocity.²⁵

In this study, we demonstrate that NR, a commercially bioavailable NAD⁺ precursor,²⁶ can increase peak I_{Na} in heterologous expression systems and RNCMs without influencing the NAD⁺/NADH ratio. We also show, for the first time, that NAD⁺ precursor supplementation reduces I_{Na,L} in RNCMs and shortens QTc in mice, collectively suggesting that both an increase in peak I_{Na} and a decrease in I_{Na,L} are novel potential antiarrhythmic mechanisms following NAD⁺ supplementation. Together, these findings provide substantial evidence for a direct role of NAD⁺ in regulating the electrical activity of the heart through modulation of Nav1.5.

Translation and trafficking of Nav1.5 to the cellular membrane surface is tightly regulated by a series of processes involving microRNA-based mRNA degradation, translation, assembly, transport through the endoplasmic reticulum and Golgi complex, anchoring at the cellular surface, recycling of the channel through internalization, and degradation.^{27, 28} A variety of

mechanisms coordinate the trafficking of Nav1.5 and involve a collection of accessory interacting proteins and post-translational modifications. At the surface, Nav1.5 function can be dictated by open probability, conductance, and gating properties. We previously reported that acetylation of Nav1.5 at a highly conserved lysine (K1479) in the Domain III-IV intracellular linker, a region of the channel historically known for its role in channel inactivation, regulates channel surface expression but not gating properties.¹⁵ However, the mechanisms regulating Nav1.5 amplitude and properties in response to NAD⁺ precursor supplementation remain unknown.

Our data here demonstrates that the application of 500 μ M to 5 mM of the NAD⁺ precursor NR increased peak I_{Na} by approximately 40-60% in HEK293 cells stably-expressing Nav1.5, HEK293 cells transiently-transfected with Nav1.5, and RNCMs with no significant change in Nav1.5 expression, channel steady-state gating properties, or channel surface and membrane expression. We also show that NR decreases K1479 Nav1.5 acetylation only at the higher 5 mM dose. Together, these findings suggest suggestive that the increase in I_{Na} observed at both doses (500 μ M and 5 mM) is independent of acetylation in heterologous cell systems are cardiac myocytes.

NAD⁺ is utilized as a substrate for sirtuins (SIRTs) and activity is dependent on NAD⁺ levels. NR drives sirtuin activity by increase NAD⁺ content. SIRT1 (Sirtuin 1), the primary and most evolutionary conserved sirtuin, deacetylates Nav1.5 at K1479 in heterologous cell systems, RNCMs, and mice.¹⁵ The enzymatic reaction of sirtuin deacetylation results in the breakdown of NAD⁺ to NAM and ADP-ribose. NAM also holds the potential to regenerate NAD⁺ but at high

doses can be a competitive feedback inhibitor on sirtuins and other NAD⁺-consuming enzymes, and we have previously shown that 5 mM NAM increases K1479 acetylation.¹⁵ In this study, the application of NAM on RNCMs demonstrated a dose-dependent effect on I_{Na}, with low doses stimulating I_{Na} but higher doses having no effect on I_{Na} despite increasing NAD⁺ content and NAD⁺/NADH ratio. This illustrates the effects of NAM in boosting NAD⁺ synthesis but inhibitory of sirtuins. NAM at either dose (500 μM or 5 mM) had no effect of I_{Na} in HEK293 cells transiently transfected with wild-type Nav1.5. Furthermore, we confirmed the importance of acetylation in modulating the response of I_{Na} to NAM using the Nav1.5 K1479A mutant. The absence of the Nav1.5-K1479 acetylation site did not significantly blunt the effect of NR on I_{Na}, suggesting that sirtuin-mediated deacetylation is not primarily responsible for the increase on I_{Na} following NR administration. However, disruption of the acetylation site enabled NAM to increase I_{Na} in HEK293 cells transiently-transfected with Nav1.5-K1479A. This illustrates that NAD⁺ metabolites can modulate Nav1.5 through both sirtuin-dependent and sirtuin-independent mechanisms.

In addition to acetylation, both protein kinase C (PKC) and protein kinase A (PKA) phosphorylation of Nav1.5 has been identified as being sensitive to NAD⁺, NADH, and metabolic stress. Increased levels of NADH activate PKC, resulting in the production of mitochondrial reactive oxygen species (ROS) and the direct phosphorylation of Nav1.5 at S1503 within the Domain III-IV linker region.¹³ These actions have been shown to decrease single channel conductance as well as decrease channel surface expression.^{13, 14} By increasing NAD⁺ content, the production of reactive oxidant species is reduced and therefore, the application of NR may modulate the PKC-mediated phosphorylation and oxidant stress production in the

regulation of Nav1.5.²⁵ Our data demonstrates that the disruption of the PKC site at S1503 abolished the effect of NR, suggesting that the effect of NR on increasing I_{Na} is dependent on the PKC phosphorylation site, which is known to decrease single channel conductance. As opposed to PKC, the stimulation of PKA phosphorylation, shown to be mediated by NAD^+ , blunts the effects of NADH on decreasing I_{Na} .^{12, 29, 30} PKA stimulation has been shown to have no effect on $I_{Na,L}$ in wild-type Nav1.5 channels.³¹ Together, the interplay between acetylation, phosphorylation, and mitigation of oxidant stress may provide an explanation of NAD^+ -mediated modulation of peak I_{Na} through the cardiac sodium channel Nav1.5 (Figure 8).

NAD^+ supplementation may also drive other unidentified post-translational modifications of Nav1.5 including ADP-ribosylation. In addition to sirtuins, NAD^+ is consumed as a substrate by poly(ADP)-ribose polymerases (PARPs) to catalyze the addition of ADP-ribose moieties onto target proteins.³² Our data illustrates that NAD^+ supplementation with NR may be stimulating ADP-ribosylation, as ADP-ribose levels are markedly depressed after NR treatment in RNCMs (Figure 5). Of note, similar decreases of ADP-ribose are not observed after NAM treatment as NAM inhibits PARP activity.³³ Investigation of ADP-ribosylation may provide further insight into the NAD^+ -dependent regulation of Nav1.5.

Previous reports have indicated that CD38 mediates the effect of extracellular NAD^+ in rescuing I_{Na} in response to increased intracellular NADH.²⁵ CD38 has been shown catalyze intracellular NAD^+ to cyclic ADP-ribose in addition to metabolizing extracellular NAD^+ precursors.³⁴ Although HEK293 cells do not express CD38, HEK293 cells and fetal bovine serum have been shown to metabolize NAD^+ precursors into other intermediates.³⁵ In our *in vitro* studies, media

and treatment was replenished every 24 hours to prevent precursor degradation over the time course of the experiment. Collectively, CD38 activity and other NAD⁺ intermediates may play additional roles in regulating the cardiac sodium channels and cardiac electrophysiology that are not yet known.

An increase in the sustained or late cardiac Na⁺ current, I_{Na,L}, seen in congenital long QT syndrome type 3 (LQT3) and in acquired disorders including cardiomyopathies, can induce a pro-arrhythmic state through prolongation of the action potential leading to early afterdepolarizations while also contributing to myocyte sodium and calcium loading.³⁶⁻³⁸ Blockers of I_{Na,L} including flecainide in LQT3 and ranolazine have been studied as potential antiarrhythmic agents.³⁹⁻⁴¹ Our data demonstrates a reduction in I_{Na,L} with NR treatment in RNCMs *in vitro* and QTc shortening after NR supplementation in mice *in vivo*. A potential mechanism of NR reducing I_{Na,L} may be mitigating oxidative stress. Oxidative stress and reactive oxygen species (ROS) decrease I_{Na} and increase I_{Na,L}, contributing to electrical and contractile dysfunction in heart failure.^{42, 43} The activation of Ca/Calmodulin-Kinase II (CaMKII) and the CaMKII-dependent phosphorylation of Nav1.5 is required for oxidative stress to increase I_{Na,L}.⁴⁴ NR supplementation and increased NAD⁺ metabolites may decrease oxidative stress and ROS, leading to lower CaMKII activity and decreased I_{Na,L}. NR may have additional effects on other ion channels and their subunits including voltage-gated K⁺ and Ca²⁺ channels that mediate repolarizing outward potassium currents and QTc.⁴⁵ We did not see changes in K⁺ or Ca²⁺ currents in RNCMs following NR supplementation that would shorten action potential duration and QTc. It is important to note the differences between mouse and human cardiac

electrophysiology as mouse action potentials are much shorter and regulated by different repolarizing K^+ currents.⁴⁶

Modulating Nav1.5 provides the therapeutic potential for preventing arrhythmias in a variety of inherited and acquired diseases associated with Nav1.5 dysfunction including Brugada Syndrome, sick sinus syndrome, progressive conduction disease, LQT3 and ischemic/nonischemic cardiomyopathies. Our data demonstrates the ability of NR to increase peak I_{Na} while reducing $I_{Na,L}$ in heterologous systems and RNCMs. The potential relevance of these findings was reinforced by QTc shortening in mice after dietary supplementation of NR. Collectively, these novel findings provide preclinical evidence and the foundation for further testing of select NAD^+ precursors for the prevention of arrhythmias in conditions associated with Nav1.5 dysfunction that decreases I_{Na} and/or increases $I_{Na,L}$.

5. CONCLUSIONS

Boosting NAD⁺ content using the NAD⁺ precursor NR but not NAM increased peak I_{Na} in heterologous expression systems and RNCMs through both acetylation-independent and acetylation-dependent mechanisms. In addition, NR supplementation decreased I_{Na,L} in RNCMs and shortened QTc in mice. These findings reinforce the potential role of the NAD⁺ supplementation in cardiac electrophysiology and warrant investigation of NR supplementation as an antiarrhythmic strategy in inherited and acquired disorders associated with abnormal Na⁺ currents.

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8. Abbreviations

Na _v 1.5	cardiac sodium channel
I _{Na}	Na ⁺ current
I _{Na,L}	late Na ⁺ current
SCN5A	cardiac sodium channel gene
PKC	Protein Kinase C
PKA	Protein Kinase A
SIRT1	Sirtuin 1
RNCM	Neonatal Rat Cardiomyocyte
meNAM	N1-methylnicotinamide
NAM	Nicotinamide
NR	Nicotinamide Riboside
LQT3	Long QT Syndrome Type 3
CD38	cluster of differentiation 38
PARP	Poly-ADP-Ribose Polymerases
NADP ⁺	nicotinamide adenine dinucleotide phosphate
NAD ⁺	β- nicotinamide adenine dinucleotide
NADH	reduced form of nicotinamide adenine dinucleotide

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Figure 1. Nicotinamide Riboside (NR) increases I_{Na} without a change in NAD⁺/NADH ratio in HEK293 cells stably-expressing and transiently-expressing Nav1.5. (A) Structure of NR, (B) Quantification of NAD⁺ and NADH demonstrating significantly elevated levels of both metabolites, without altering the NAD⁺/NADH ratio, with NR supplementation (C) Representative traces of I_{Na}, (D) current-voltage (I-V) relationship, and (E) normalized peak current density between HEK293 cells stably-expressing Nav1.5 with and without NR supplementation (500 μM, 48 hours) illustrate that NR increases I_{Na}. (F) Gating properties including steady-state activation and inactivation are relatively unchanged, with only a 4 mV hyperpolarizing shift in activation, with NR supplementation. (G-I) Similar results are observed in HEK293 cells transiently-transfected with Nav1.5 (5 mM NR, 48 hours). Differences between groups were statistically determined utilizing unpaired t-tests (*p<0.05, *p<0.001).**

Figure 2. Nicotinamide Riboside (NR) increases I_{Na} with dose-dependent effects on gating properties in Neonatal Rat Cardiomyocytes. (A) Representative current traces, (B) I-V curve, and (C) normalized peak current density of rat myocytes treated with NR for 48 hours. (D) Gating properties of steady-state activation and inactivation (E-F) Representative traces and quantification of $I_{Na,L}$ (G-I) Representative traces and quantification of tetrodotoxin (TTX)-sensitive changes in $I_{Na,L}$. (I) NAD⁺, NADH, and NAD⁺/NADH ratio quantified in rat myocytes after 24-hour NR treatment (500 μ M or 5 mM). Differences between groups were statistically determined utilizing unpaired t-tests (*p<0.05, **p<0.01, ***p<0.001).

Figure 3. Nicotinamide Riboside supplementation decreases K1479 acetylation in Nav1.5-HEK293 cells. (A) Acetylated K1479 Nav_v1.5, total Nav_v1.5, and GAPDH expression in HEK293 cells transiently-transfected cells Nav_v1.5 and treated with NR (500 μ M or 5 mM, 48 hours) or H₂O. (B) Acetylated K1479 was normalized to total Nav_v1.5 expression and (C) total Nav_v1.5 expression was normalized to GAPDH expression. Differences between groups were statistically determined utilizing One-Way ANOVA tests with multiple comparisons (*p<0.05).

Figure 4. Nicotinamide (NAM), an alternative NAD^+ precursor, does not modulate Nav1.5 in HEK293 cells transiently transfected with Nav1.5 but dose-dependently modulates Nav1.5 in RNCMs (A) Structure of NAM, (B) I-V curve, and (C) normalized peak current density of HEK293 cells transiently transfected with Nav1.5 and administered NAM (500 μ M or 5 mM, 48 hours). (D, E) The effect of NAM supplementation (500 μ M or 5 mM, 24 hours) on I-V curve and normalized peak current density illustrates dose-dependent effects on Nav1.5 activity in RNCMs. 500 μ M NAM stimulates I_{Na} whereas 5 mM NAM has no effect on I_{Na} . (F) Gating properties of steady-state activation and inactivation. (G) Late current, $I_{Na,L}$, in RNCMs treated with NAM (H) NAD^+ , $NADH$, and $NAD^+/NADH$ ratio quantified in RNCMs after 24-hour NAM treatment. Differences between groups were statistically determined utilizing unpaired t-tests (*p<0.05, **p<0.01, *p<0.001).**

Figure 5. NAD⁺ precursors Nicotinamide Riboside (NR) and Nicotinamide (NAM) increases NAD⁺ Content in Neonatal Rat Cardiomyocytes. (A-I) NAD⁺, NADP, NAAD, NMN, NAM, NR, NAR, meNAM, and ADPR were assessed by LC-MS in cellular extracts of neonatal rat cardiomyocytes treated with either H₂O, NR, NAM, or meNAM (500 μM or 5 mM, 24 hours). Differences between groups were statistically determined utilizing unpaired t-tests (*p<0.05, **p<0.01, ***p<0.001 vs. respective groups and †p<0.05, ‡p<0.05, §p<0.001 vs. control).

Figure 6. The Role of the Nav1.5 Acetylation K1479 and Phosphorylation S1503 sites in mediating the effect of NAD⁺ precursors. The I-V relationship, peak current density, and steady-state activation and inactivation of HEK293 cells transiently transfected with: (A-C) the mutant Nav1.5 K1479A channel with and without the administration of NR (5 mM, 48 hours), (D-F) the mutant Nav1.5 K1479A/S1503A channel with and without the administration of NR (5 mM, 48 hours), and (G-I) the mutant Nav1.5 K1479A channel with and without the administration of NAM (500 μ M or 5 mM, 48 hours). Differences between normalized peak current densities were statistically determined utilizing unpaired t-tests (*p<0.05).

Figure 7. Dietary Supplementation of Nicotinamide Riboside Modulates Cardiac Repolarization. 4-6 month old wild-type C57BL/6 mice were randomly placed on either a control diet or diet supplemented with NR (0.35% or 1%). Cardiac electrophysiological changes were assessed by EKG. **(A, B)** Representative Lead II EKG traces overlaying baseline (dashed line) and 10-12 weeks post-diet (solid line) for mice on either control diet or diet supplemented with 1.0% NR. **(C)** PR interval, **(D)** QRS interval, and **(E)** QTc duration were measured. Significance between groups and within a group at each timepoint was determined by Two-way ANOVA with Bonferroni post-hoc multiple-comparisons test. (*p<0.05).

Figure 8. Model of NAD⁺ Precursor Modulation of Nav1.5.

SUPPLEMENTARY DATA

Figure S1. The Effect of Nicotinamide Riboside (NR) on $\text{Na}_v1.5$ Gating Properties in HEK293 Cells. $\text{Na}_v1.5$ Gating properties (including half-maximal potentials and slope factors) for steady-state activation and inactivation for **(Top)** HEK293 cells stably-expressing $\text{Na}_v1.5$ and treated with 500 μM for 48 hours and **(Bottom)** HEK293 cells transiently transfected with $\text{Na}_v1.5$ and treated with 5 mM NR for 48 hours. Differences between groups were statistically determined utilizing unpaired t-tests (* $p < 0.05$).

Membrane Compartment Enrichment: Expression of Na_v1.5

Figure S2. The Effect of Nicotinamide Riboside (NR) on Membrane Expression of Na_v1.5 in HEK293 Cells stably-expressing Na_v1.5. HEK293 cells stably expressing Na_v1.5 were treated with NR (500 μM or 5 mM, 48 hours) and the membrane-enriched protein fraction was harvested with the Compartmentalization Protein Extraction Kit (Millipore, Burlington, MA). Membrane-enriched Na_v1.5 was blotted, quantified, and normalized to the stain-free gel image obtained prior to transfer to a polyvinylidene fluoride (PVDF) membrane.

Control
FLAG-Nav_v1.5 HEK293

5 mM NR
FLAG-Nav_v1.5 HEK293

HEK293 Control

Figure S3. The Effect of Nicotinamide Riboside (NR) on Surface Expression of FLAG-Nav_v1.5. HEK293 cells stably-expressing Nav_v1.5 with a FLAG tag in the extracellular region of D1 was treated with 5 mM NR for 48 hours (replenished at 24 hours) and assessed for surface expression by confocal microscopy. HEK293 cells without Nav_v1.5 were used as a control.

Figure S4. The Effect of Nicotinamide Riboside (NR) on Normalized I_{Na} Gating Properties in RNCMs. (Top) Steady-state activation and (Bottom) inactivation properties of neonatal rat cardiomyocytes (RNCMs) treated with H₂O (vehicle) or NR at either 500 μ M or 5 mM for 24 hours. Myocyte properties were normalized to vehicle litter-controlled myocytes to account for litter-to-litter variation in harvesting myocytes. Differences between groups were statistically determined utilizing unpaired t-tests (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure S5. The Effect of Nicotinamide Riboside (NR) on Late Sodium Current Density in RNCMs at . Differences between groups were statistically determined utilizing unpaired t-tests (** $p < 0.001$).

Figure S6. The Effect of Nicotinamide Riboside (NR) on *Scn5a* transcript expression in Neonatal Rat Cardiomyocytes. *Scn5a* transcript expression (normalized to beta-actin expression) was unchanged with administration of 500 μ M NR for 24 hours (Ctrl: 1.00 ± 0.05 vs. NR: 1.079 ± 0.05 , $p=0.33$ unpaired t-test).

Figure S7. NR has no effect on I_K and I_{Ca} Currents. (A-B) Whole-cell current-voltage relationships measured at 10 ms into depolarization (I_{Ktotal}) and just prior to repolarization to measure the sustained I_K (I_{Ksus}) recorded from rat cardiomyocytes in control or NR-treated (5 mM, 24 hours) myocytes. (C-D) Current-voltage relationship of transient outward K^+ current (I_{to}) and ultra-rapid K^+ current (I_{Kur}). No statistically difference between control and NR-treated cells was observed at various membrane potentials. (E) Current-voltage relationships for calcium currents recorded from neonatal rat cardiomyocytes. (F) The peak current density and (G) inactivation time constant (τ) estimated from exponential fits to currents measured at 0 mV recorded from cells control or NR-treated (5 mM NR, 24 hours). Values are mean $\pm$ SE.

Figure S8. The Effect of Nicotinamide (NAM) on Nav1.5 Gating Properties in HEK293 Cells. (Top) Gating properties of steady-state activation and inactivation demonstrate are unchanged with NAM treatment. **(Bottom)** Nav1.5 gating properties (including half-maximal potentials and slope factors) for steady-state activation and inactivation of HEK293 cells transiently transfected with Nav1.5 and treated with H₂O (vehicle), 500 μ M NAM, or 5mM NAM for 48 hours.

Figure S9. The Effect of Nicotinamide (NAM) on I_{Na} Gating Properties in RNCMs. Steady-state activation and inactivation properties of neonatal rat cardiomyocytes (NRCMs) treated with H_2O (vehicle) or NAM at either 500 μ M or 5 mM for 24 hours. Myocyte properties were normalized to vehicle litter-controlled myocytes to account for litter-to-litter variation in harvesting myocytes.

Figure S10. Inert NAD⁺ Metabolite 1-Methyl-Nicotinamide has no effect on I_{Na} in Neonatal Rat Cardiomyocytes. (A) The structure of 1-methyl-Nicotinamide (meNAM), an inert byproduct of Nicotinamide which cannot be utilized for NAD⁺ synthesis. (B,C,D) meNAM has no to minimal effects on I_{Na} indicated by I-V curve, normalized peak current density, and steady-state activation and inactivation curves. (E-F) Gating properties are unchanged after treatment with meNAM. (G) Late I_{Na} was unaffected with meNAM supplementation. (H) NAD⁺, NADH, and NAD⁺/NADH ratio quantified in rat myocytes after 24-hour meNAM treatment.

Figure S11. Electrophysiological Comparison of the Wild-type and Mutant K1479A $Na_v1.5$ Channel. (A-C) The I-V relationship, peak current density, and steady-state activation and inactivation of HEK293 cells transiently transfected with either the wild-type or mutant $Na_v1.5$ K1479A channel. **(D-E)** $Na_v1.5$ gating properties (including half-maximal potentials and slope factors) for steady-state activation and inactivation of HEK293 cells transiently transfected with wild-type and K1479A $Na_v1.5$. **(F)** Comparison of late I_{Na} between wild-type and K1479A $Na_v1.5$ as measured by the 10 ms average current at 200 ms post-depolarization at -20 mV.

NR on HEK 293 Cells Transiently Transfected with Na_v1.5 K1479A

NAM on HEK 293 Cells Transiently Transfected with Na_v1.5 K1479A

Figure S12. The Effect of Nicotinamide Riboside (NR) and Nicotinamide (NAM) on Na_v1.5 K1479A Gating Properties in HEK293 Cells. Na_v1.5 gating properties (including half-maximal potentials and slope factors) for steady-state activation and inactivation of HEK293 cells transiently transfected with Na_v1.5 K1479A and treated with **(Top)** H₂O (vehicle) or 5 mM NR or **(Bottom)** H₂O (vehicle), 500 μM NAM, or 5 mM NAM for 48 hours.

Figure S13. The Effect of Nicotinamide Riboside (NR) dietary supplementation on body weight in C57BL/6 wild-type mice. Body weights were assessed throughout the time course of NR supplementation. No changes in weight were observed between groups throughout the course of the experiment (Two-way ANOVA Bonferroni multiple comparisons post-hoc test).

HEK293 Cells Stably Expressing Nav1.5

Control (H ₂ O)				500 μM NR				
<i>n</i>	Peak Current Density (pA/pF)	Peak Potential (mV)	Normalized Current Density	<i>n</i>	Peak Current Density (pA/pF)	Peak Potential (mV)	Normalized Current Density	
7	-292.9 ± 38	-7.85 ± 1.8	-1.00 ± 0.13	6	-370.4 ± 116	-15.83 ± 4.7	-1.26 ± 0.39	
6	-152.3 ± 49	-12.50 ± 2.5	-1.00 ± 0.32	7	-237.2 ± 44	-11.43 ± 2.3	-1.55 ± 0.29	
6	-188.9 ± 63	-9.16 ± 3.0	-1.00 ± 0.33	8	-296.6 ± 77	-16.25 ± 2.6	-1.57 ± 0.40	
5	-83.9 ± 20	-7.00 ± 2.0	-1.00 ± 0.24	7	-135.5 ± 33	-7.85 ± 1.4	-1.61 ± 0.40	
Total/Average	24	-195.6 ± 26	-9.16 ± 1.1	-1.00 ± 0.12	28	-257.3 ± 37	-12.86 ± 1.5	-1.51 ± 0.18

Unpaired t-test

Peak Current Density: Control (H₂O) vs. 500 μ M NR $p = 0.19$
 Peak Potential: Control (H₂O) vs. 500 μ M NR $p = 0.06$
 Normalized Peak Current Density: Control (H₂O) vs. 500 μ M NR $p = 0.02$

HEK293 Cells Transiently Expressing Nav1.5

Control (H ₂ O)				5 mM NR				
<i>n</i>	Peak Current Density (pA/pF)	Peak Potential (mV)	Normalized Current Density	<i>n</i>	Peak Current Density (pA/pF)	Peak Potential (mV)	Normalized Current Density	
4	-63.1 ± 22	-7.50 ± 4.3	-1.00 ± 0.35	7	-116.5 ± 28	-8.57 ± 4.7	-1.87 ± 0.45	
9	-112.7 ± 39	-14.44 ± 2.2	-1.00 ± 0.34	7	-99.6 ± 10	-15.71 ± 1.7	-0.89 ± 0.17	
4	-11.7 ± 3	-7.50 ± 3.2	-1.00 ± 0.32	4	-25.0 ± 6	-8.75 ± 4.2	-2.13 ± 0.57	
5	-31.6 ± 10	-2.00 ± 4.6	-1.00 ± 0.31	6	-60.2 ± 23	-3.33 ± 2.7	-1.90 ± 0.74	
5	-61.2 ± 22	-17.00 ± 2.5	-1.00 ± 0.36	5	-88.2 ± 26	-14.00 ± 1.8	-1.43 ± 0.42	
Total/Average	27	-65.8 ± 15	-10.56 ± 1.7	-1.00 ± 0.15	29	-83.2 ± 10	-10.17 ± 1.6	-1.59 ± 0.22

Unpaired t-test

Peak Current Density: Control (H₂O) vs. 5 mM NR $p = 0.35$
 Peak Potential: Control (H₂O) vs. 5 mM NR $p = 0.87$
 Normalized Peak Current Density: Control (H₂O) vs. 5 mM NR $p = 0.03$

Table S1. Current Densities, Peak Potentials, and Normalized Current Densities for HEK293 cells stably and transiently expressing Nav1.5 treated with Nicotinamide Riboside (NR).

Neonatal Rat Cardiomyocytes

Control (H ₂ O)				500 μ M NR				5 mM NR				
<i>n</i>	Peak Current Density (pA/pF)	Peak Potential (mV)	Normalized Current Density	<i>n</i>	Peak Current Density (pA/pF)	Peak Potential (mV)	Normalized Current Density	<i>n</i>	Peak Current Density (pA/pF)	Peak Potential (mV)	Normalized Current Density	
10	-130.8 $\pm$ 21	-39.0 $\pm$ 1.6	-1.00 $\pm$ 0.16	13	-209.4 $\pm$ 34	-39.6 $\pm$ 1.3	-1.60 $\pm$ 0.26	2	-145.2 $\pm$ 40	-27.5 $\pm$ 2.5	-1.48 $\pm$ 0.41	
3	-97.6 $\pm$ 38	-38.3 $\pm$ 1.6	-1.00 $\pm$ 0.39					6	-181.9 $\pm$ 58	-33.3 $\pm$ 1.6	-1.09 $\pm$ 0.35	
4	-166.9 $\pm$ 37	-35.0 $\pm$ 2.0	-1.00 $\pm$ 0.22					4	-168.2 $\pm$ 50	-30.0 $\pm$ 3.5	-1.72 $\pm$ 0.86	
2	-58.7 $\pm$ 15	-45.0 $\pm$ 5.0	-1.00 $\pm$ 0.26									
Total/Average	19	-125.6 $\pm$ 15	-38.6 $\pm$ 1.2	-1.00 $\pm$ 0.10	13	-209.4 $\pm$ 34	-39.6 $\pm$ 1.3	-1.60 $\pm$ 0.26	12	-171.2 $\pm$ 32	-31.2 $\pm$ 1.5	-1.74 $\pm$ 0.39

Unpaired t-test

Peak Current Density: Control (H ₂ O) vs. 500 μ M NR	$p = 0.02$
Peak Potential: Control (H ₂ O) vs. 500 μ M NR	$p = 0.61$
Normalized Peak Current Density: Control (H ₂ O) vs. 500 μ M NR	$p = 0.02$
Peak Current Density: Control (H ₂ O) vs. 5 mM NR	$p = 0.17$
Peak Potential: Control (H ₂ O) vs. 5 mM NR	$p = 0.0006$
Normalized Peak Current Density: Control (H ₂ O) vs. 5 mM NR	$p = 0.03$

Table S2. Current Densities, Peak Potentials, and Normalized Currents for Neonatal Rat Cardiomyocytes treated with Nicotinamide Riboside (NR).

HEK293 Cells Transiently Expressing Nav1.5

Control (H ₂ O)				500 μ M NAM				5 mM NAM							
<i>n</i>	Peak Current Density (pA/pF)	Peak Potential (mV)	Normalized Current Density	<i>n</i>	Peak Current Density (pA/pF)	Peak Potential (mV)	Normalized Current Density	<i>n</i>	Peak Current Density (pA/pF)	Peak Potential (mV)	Normalized Current Density				
4	-72.8 $\pm$ 29.7	-3.7 $\pm$ 3.1	-1.00 $\pm$ 0.40	4	-21.4 $\pm$ 4.7	-2.5 $\pm$ 5.2	-0.29 $\pm$ 0.06	6	-73.8 $\pm$ 22.9	-6.6 $\pm$ 5.4	-1.01 $\pm$ 0.31				
4	-34.3 $\pm$ 7.5	-2.5 $\pm$ 2.5	-1.00 $\pm$ 0.22	6	-18.6 $\pm$ 6.3	-7.5 $\pm$ 5.5	-0.54 $\pm$ 0.18	4	-46.4 $\pm$ 26.2	-11.2 $\pm$ 6.8	-1.35 $\pm$ 0.82				
6	-61.5 $\pm$ 8.6	-0.0 $\pm$ 5.3	-1.00 $\pm$ 0.14	7	-82.2 $\pm$ 35.3	-1.4 $\pm$ 3.2	-1.33 $\pm$ 0.57	5	-82.1 $\pm$ 25.7	-4.0 $\pm$ 4.8	-1.33 $\pm$ 0.41				
Total/Average				14	-56.9 $\pm$ 9.6	-1.7 $\pm$ 2.4	-1.00 $\pm$ 0.13	17	-45.5 $\pm$ 16.0	-2.6 $\pm$ 2.6	-0.81 $\pm$ 0.26	15	-69.3 $\pm$ 14.0	-7.0 $\pm$ 3.1	-1.21 $\pm$ 0.26

Unpaired t-test

Peak Current Density: Control (H ₂ O) vs. 500 μ M NAM	p = 0.56
Peak Potential: Control (H ₂ O) vs. 500 μ M NAM	p = 0.81
Normalized Peak Current Density: Control (H ₂ O) vs. 500 μ M NAM	p = 0.56
Peak Current Density: Control (H ₂ O) vs. 5 mM NAM	p = 0.48
Peak Potential: Control (H ₂ O) vs. 5 mM NAM	p = 0.20
Normalized Peak Current Density: Control (H ₂ O) vs. 5 mM NAM	p = 0.49

Neonatal Rat Cardiomyocytes

Control (H ₂ O)				500 μ M NAM				5 mM NAM				
<i>n</i>	Peak Current Density (pA/pF)	Peak Potential (mV)	Normalized Current Density	<i>n</i>	Peak Current Density (pA/pF)	Peak Potential (mV)	Normalized Current Density	<i>n</i>	Peak Current Density (pA/pF)	Peak Potential (mV)	Normalized Current Density	
8	-93.2 $\pm$ 23.2	-36.8 $\pm$ 0.9	-1.00 $\pm$ 0.24	7	-197.9 $\pm$ 45.9	-35.7 $\pm$ 2.0	-2.12 $\pm$ 0.49					
4	-116.4 $\pm$ 32.4	-37.5 $\pm$ 2.5	-1.00 $\pm$ 0.27	4	-158.1 $\pm$ 31.3	-37.5 $\pm$ 3.2	-1.35 $\pm$ 0.26	7	-207.3 $\pm$ 73.7	-37.8 $\pm$ 1.8	-1.04 $\pm$ 0.37	
7	-198.0 $\pm$ 23.1	-40.0 $\pm$ 1.8	-1.00 $\pm$ 0.11					5	-104.3 $\pm$ 29.2	-39.0 $\pm$ 2.4	-0.61 $\pm$ 0.17	
6	-170.6 $\pm$ 32.0	-35.0 $\pm$ 1.8	-1.00 $\pm$ 0.18									
Total/Average	25	-144.9 $\pm$ 15.5	-37.4 $\pm$ 0.8	-1.00 $\pm$ 0.10	11	-183.4 $\pm$ 30.8	-36.3 $\pm$ 1.6	-1.84 $\pm$ 0.33	12	-164.4 $\pm$ 45.7	-38.3 $\pm$ 1.4	-0.86 $\pm$ 0.22

Unpaired t-test

Peak Current Density: Control (H ₂ O) vs. 500 μ M NAM	p = 0.22
Peak Potential: Control (H ₂ O) vs. 500 μ M NAM	p = 0.54
Normalized Peak Current Density: Control (H ₂ O) vs. 500 μ M NAM	p = 0.003
Peak Current Density: Control (H ₂ O) vs. 5 mM NAM	p = 0.61
Peak Potential: Control (H ₂ O) vs. 5 mM NAM	p = 0.56
Normalized Peak Current Density: Control (H ₂ O) vs. 5 mM NAM	p = 0.53

Table S3. Current Densities, Peak Potentials, and Normalized Currents for HEK293 cells transiently expressing Nav1.5 and Neonatal Rat Cardiomyocytes treated with Nicotinamide (NAM).

HEK293 Cells Transiently Expressing Nav1.5 K1479A

Control (H ₂ O)				5 mM NR				
n	Peak Current Density (pA/pF)	Peak Potential (mV)	Normalized Current Density	n	Peak Current Density (pA/pF)	Peak Potential (mV)	Normalized Current Density	
11	-94.6 ± 38	-4.54 ± 2.8	-1.00 ± 0.31	10	-155.5 ± 60	-11.50 ± 2.8	-1.64 ± 0.63	
5	-89.4 ± 29	-5.00 ± 6.3	-1.00 ± 0.33	5	-96.7 ± 21	-10.00 ± 4.1	-1.08 ± 0.24	
4	-26.3 ± 6	3.75 ± 3.7	-1.00 ± 0.23	6	-56.9 ± 17	-6.66 ± 3.8	-2.16 ± 0.67	
4	-11.1 ± 3	-8.75 ± 4.2	-1.00 ± 0.31	4	-16.3 ± 0.9	-11.25 ± 5.1	-1.46 ± 0.08	
6	-31.6 ± 15	-17.50 ± 3.8	-1.00 ± 0.29	4	-130.2 ± 52	-18.75 ± 3.1	-2.52 ± 1.01	
Total/Average	30	-54.8 ± 13	-6.66 ± 2.0	-1.00 ± 0.14	29	-84.6 ± 4	-11.21 ± 1.6	-1.75 ± 0.29

Unpaired t-test

Peak Current Density: Control (H₂O) vs. 5 mM NR

p = 0.27

Peak Potential: Control (H₂O) vs. 5 mM NR

p = 0.09

Normalized Peak Current Density: Control (H₂O) vs. 5 mM NR

p = 0.02

HEK293 Cells Transiently Expressing Nav1.5 K1479A/S1503A

Control (H ₂ O)				5 mM NR				
n	Peak Current Density (pA/pF)	Peak Potential (mV)	Normalized Current Density	n	Peak Current Density (pA/pF)	Peak Potential (mV)	Normalized Current Density	
10	-46.0 ± 12	4.50 ± 1.1	-1.00 ± 0.26	10	-49.0 ± 6	-1.50 ± 1.6	-1.06 ± 0.13	
7	-52.4 ± 13	0.00 ± 2.4	-1.00 ± 0.25	8	-72.6 ± 13	-0.62 ± 1.1	-1.38 ± 0.24	
7	-32.7 ± 7	4.28 ± 2.2	-1.00 ± 0.24	7	-34.7 ± 9	0.71 ± 5.1	-1.05 ± 0.29	
Total/Average	24	-44.0 ± 6	3.12 ± 1.1	-1.00 ± 0.14	25	-52.6 ± 6	-0.60 ± 1.5	-1.16 ± 0.12

Unpaired t-test

Peak Current Density: Control (H₂O) vs. 5 mM NR

p = 0.35

Peak Potential: Control (H₂O) vs. 5 mM NR

p = 0.06

Normalized Peak Current Density: Control (H₂O) vs. 5 mM NR

p = 0.38

HEK293 Cells Transiently Expressing Nav1.5 K1479A

Control (H ₂ O)				500 μM NAM				5 mM NAM			
<i>n</i>	Peak Current Density (pA/pF)	Peak Potential (mV)	Normalized Current Density	<i>n</i>	Peak Current Density (pA/pF)	Peak Potential (mV)	Normalized Current Density	<i>n</i>	Peak Current Density (pA/pF)	Peak Potential (mV)	Normalized Current Density
8	-47.4 ± 11	-3.75 ± 3.2	-1.00 ± 0.24	8	-92.1 ± 34	0.00 ± 2.9	-1.94 ± 0.72	7	-72.5 ± 17	-3.57 ± 4.1	-1.52 ± 0.37
6	-36.6 ± 12	2.00 ± 2.5	-1.00 ± 0.33	4	-56.9 ± 6	1.23 ± 2.3	-1.55 ± 0.18	8	-65.5 ± 13	-3.75 ± 2.4	-1.78 ± 0.36
Total/Average				14	-80.3 ± 23	0.41 ± 2.0	-1.81 ± 0.47	15	-80.3 ± 23	-3.66 ± 2.2	-1.66 ± 0.25

Unpaired t-test

Peak Current Density: Control (H₂O) vs. 500 μM NAM

p = 0.11

Peak Potential: Control (H₂O) vs. 500 μM NAM

p = 0.53

Normalized Peak Current Density: Control (H₂O) vs. 500 μM NAM

p = 0.10

Peak Current Density: Control (H₂O) vs. 5 mM NAM

p = 0.06

Peak Potential: Control (H₂O) vs. 5 mM NAM

p = 0.51

Normalized Peak Current Density: Control (H₂O) vs. 5 mM NAM

p = 0.04

Table S4. Current Densities, Peak Potentials, and Normalized Currents for HEK293 cells transiently expressing mutant forms (K1479A and K1479A/S1503A) and treated with either Nicotinamide Riboside (NR) or Nicotinamide (NAM).

HEK293 Cells Stably Expressing Nav1.5			Activation Parameters		Inactivation Parameters	
Treatment Group	n	Peak I_{Na} (Normalized Current)	Half Value (mV)	Slope (k)	Half Value (mV)	Slope (k)
Control (H ₂ O)	24	-1.00 ± 0.12	-20.04 ± 1.38	8.89 ± 0.35	-78.44 ± 1.78	-7.31 ± 0.23
500 μM NR	28	-1.51 ± 0.18	-24.25 ± 1.33	8.73 ± 0.36	-83.19 ± 2.22	-7.10 ± 0.16
HEK293 Cells Transiently Expressing Nav1.5			Activation Parameters		Inactivation Parameters	
Treatment Group	n	Peak I_{Na} (Normalized Current)	Half Value (mV)	Slope (k)	Half Value (mV)	Slope (k)
Control (H ₂ O)	27	-1.00 ± 0.15	-22.15 ± 2.35	8.89 ± 0.35	-83.66 ± 1.65	-6.18 ± 0.22
5 mM NR	29	-1.59 ± 0.22	-22.07 ± 1.64	8.73 ± 0.36	-83.42 ± 1.47	-6.39 ± 0.16
HEK293 Cells Transiently Expressing Nav1.5			Activation Parameters		Inactivation Parameters	
Treatment Group	n	Peak I_{Na} (Normalized Current)	Half Value (mV)	Slope (k)	Half Value (mV)	Slope (k)
Control (H ₂ O)	14	-1.00 ± 0.13	-12.23 ± 2.16	9.06 ± 0.26	-77.72 ± 3.35	-6.45 ± 0.40
500 μM NAM	17	-0.81 ± 0.26	-12.80 ± 2.42	8.56 ± 0.22	-74.51 ± 3.13	-7.42 ± 0.42
5 mM NAM	15	-1.21 ± 0.26	-18.42 ± 2.45	8.77 ± 0.34	-80.78 ± 2.79	-6.46 ± 0.37
Rat Cardiomyocytes			Normalized Activation Parameters		Normalized Inactivation Parameters	
Treatment Group	n	Peak I_{Na} (Normalized Current)	Half Value (mV)	Slope	Half Value (mV)	Slope
Control (H ₂ O)	19	-1.00 ± 0.10	-49.17 ± 1.31	8.01 ± 0.38	-97.49 ± 1.00	-6.75 ± 0.33
500 μM NR	13	-1.60 ± 0.26	-51.31 ± 1.16	6.93 ± 0.314	-96.94 ± 0.83	-7.31 ± 0.33
5 mM NR	12	-1.74 ± 0.39	-42.36 ± 0.97	8.03 ± 0.18	-92.91 ± 2.06	-9.27 ± 0.29
Treatment Group	n	Peak I_{Na} (Normalized Current)	Half Value (mV)	Slope	Half Value (mV)	Slope
Control (H ₂ O)	25	-1.00 ± 0.10	-47.83 ± 1.47	8.62 ± 0.37	-96.98 ± 1.37	-6.50 ± 0.27
500 μM NAM	11	-1.84 ± 0.33	-48.19 ± 1.92	7.93 ± 0.26	-94.76 ± 2.43	-6.91 ± 0.30
5 mM NAM	12	-0.86 ± 0.22	-50.26 ± 2.74	7.05 ± 0.79	-101.0 ± 2.60	-6.05 ± 0.93
Treatment Group	n	Peak I_{Na} (Normalized Current)	Half Value (mV)	Slope	Half Value (mV)	Slope
Control (H ₂ O)	12	-1.00 ± 0.11	-49.21 ± 1.77	7.75 ± 0.18	-95.15 ± 1.65	-6.96 ± 0.24
500 μM meNAM	6	-0.93 ± 0.14	-53.54 ± 2.36	7.75 ± 0.26	-99.18 ± 2.38	-6.56 ± 0.51
5 mM meNAM	9	-0.95 ± 0.09	-45.19 ± 2.01	7.79 ± 0.21	-91.47 ± 1.37	-6.35 ± 0.15
HEK293 Cells Transiently Expressing Nav1.5 K1479A						
Treatment Group	n	Peak I_{Na} (Normalized Current)	Half Value (mV)	Slope	Half Value (mV)	Slope
Control (H ₂ O)	29	-1.00 ± 0.14	-13.70 ± 2.38	8.72 ± 0.32	-81.47 ± 2.73	-7.51 ± 0.60
5 mM NR	30	-1.75 ± 0.29	-21.29 ± 1.93	8.34 ± 0.18	-90.42 ± 1.81	-6.68 ± 0.20
HEK293 Cells Transiently Expressing Nav1.5 K1479A/S1503A						
Treatment Group	n	Peak I_{Na} (Normalized Current)	Half Value (mV)	Slope	Half Value (mV)	Slope
Control (H ₂ O)	24	-1.00 ± 0.14	-7.89 ± 1.22	8.73 ± 0.19	-75.43 ± 1.03	-7.33 ± 0.27
5 mM NR	25	-1.16 ± 0.12	-11.83 ± 1.45	9.07 ± 0.23	-79.08 ± 1.21	-7.39 ± 0.20
HEK293 Cells Transiently Expressing Nav1.5 K1479A						
Treatment Group	n	Peak I_{Na} (Normalized Current)	Half Value (mV)	Slope	Half Value (mV)	Slope
Control (H ₂ O)	14	-1.00 ± 0.19	-11.81 ± 1.73	9.13 ± 0.25	-82.03 ± 1.73	-6.82 ± 0.33
500 μM NAM	12	-1.81 ± 0.47	-11.51 ± 2.91	9.13 ± 0.20	-79.39 ± 3.33	-7.35 ± 0.29
5 mM NAM	15	-1.66 ± 0.25	-15.41 ± 2.20	9.44 ± 0.27	-83.63 ± 2.72	-6.51 ± 0.37

Table S5. Gating Properties for Whole Cell Patch Clamp Experiments in HEK293 cells and Neonatal Rat Cardiomyocytes.

***In Vivo* Cardiac Electrophysiology Data**

Baseline						
	Heart Rate Lead I (bpm)	Heart Rate QT Measurement (bpm)	PR Interval (ms)	QRS Duration (ms)	QT (ms)	QTc (ms)
Control Diet (n=12)	530 ± 9	498 ± 11	46.7 ± 1.4	15.0 ± 0.2	94.2 ± 2.8	85.3 ± 2.1
0.35% NR Diet (n=7)	539 ± 24	510 ± 21	49.1 ± 3.4	14.9 ± 0.5	103.0 ± 3.9	93.9 ± 2.3
1.0% NR Diet (n=5)	552 ± 30	516 ± 18	46.8 ± 1.3	14.7 ± 0.4	88.1 ± 3.0	81.6 ± 2.8
5-6 Weeks Post-Diet						
	Lead I Heart Rate (bpm)	Heart Rate (bpm)	PR Interval (ms)	QRS Duration (ms)	QT (ms)	QTc (ms)
Control Diet (n=12)	534 ± 15	492 ± 10	47.6 ± 1.9	14.7 ± 0.3	92.7 ± 2.0	83.9 ± 2.0
0.35% NR Diet (n=7)	537 ± 14	502 ± 11	45.8 ± 2.2	14.5 ± 0.5	99.1 ± 2.3	90.4 ± 1.8
1.0% NR Diet (n=5)	566 ± 21	535 ± 32	46.6 ± 2.7	13.7 ± 0.2	85.6 ± 3.9	79.8 ± 2.1
10-12 Weeks Post-Diet						
	Lead I Heart Rate (bpm)	Heart Rate (bpm)	PR Interval (ms)	QRS Duration (ms)	QT (ms)	QTc (ms)
Control Diet (n=12)	532 ± 12	516 ± 11	45.9 ± 1.6	14.5 ± 0.3	94.0 ± 2.4	86.9 ± 1.9
0.35% NR Diet (n=7)	558 ± 16	526 ± 11	48.0 ± 2.6	15.2 ± 0.4	95.6 ± 2.4	89.3 ± 1.8
1.0% NR Diet (n=5)	502 ± 35	476 ± 24	42.3 ± 1.1	14.8 ± 0.4	83.4 ± 4.0	73.8 ± 2.2

Table S6. Effect of Nicotinamide Riboside on Cardiac Electrophysiology in C57BL/6 wild-type mice.

Echocardiographic Assessment

	Baseline						
	Heart Rate (beats/min)	EDV (μ L)	ESV (μ L)	Mass (mg)	SV (μ L)	CO (μ L/min)	EF (%)
Control Diet (n=12)	679 $\pm$ 9.	27.4 $\pm$ 2.1	5.3 $\pm$ 0.5	74.9 $\pm$ 4.4	22.1 $\pm$ 2.2	14888 $\pm$ 1399	79 $\pm$ 3
0.35% NR Diet (n=7)	701 $\pm$ 12	23.7 $\pm$ 3.7	4.3 $\pm$ 0.6	70.6 $\pm$ 2.9	18.3 $\pm$ 3.9	12802 $\pm$ 2727	73 $\pm$ 6
1.0% NR Diet (n=5)	686 $\pm$ 19	32.5 $\pm$ 3.7	5.3 $\pm$ 0.6	82.1 $\pm$ 5.4	28.2 $\pm$ 2.9	19352 $\pm$ 2041	87 $\pm$ 2
	10-12 Weeks						
	Heart Rate (beats/min)	EDV (μ L)	ESV (μ L)	Mass (mg)	SV (μ L)	CO (μ L/min)	EF (%)
Control Diet (n=12)	691 $\pm$ 10	25.4 $\pm$ 1.5	4.9 $\pm$ 0.5	72.5 $\pm$ 3.4	20.4 $\pm$ 1.3	14095 $\pm$ 928	80 $\pm$ 2
0.35% NR Diet (n=7)	637 $\pm$ 46	24.8 $\pm$ 2.4	4.9 $\pm$ 0.7	72.5 $\pm$ 3.1	19.8 $\pm$ 2.3	12259 $\pm$ 1418	79 $\pm$ 3
1.0% NR Diet (n=5)	675 $\pm$ 16	28.4 $\pm$ 5.9	5.2 $\pm$ 1.3	77.0 $\pm$ 9.1	19.8 $\pm$ 2.3	15699 $\pm$ 3276	82 $\pm$ 2

Table S7. Effect of Nicotinamide Riboside on Cardiac Function in C57BL/6 wild-type mice. Heart Rate, End-diastolic volume (EDV), End-systolic volume (ESV), Mass, Stroke volume (SV), Cardiac Output (CO), Ejection Fraction (EF).