Published in final edited form as:
Circ Res. 2010 October 15; 107(8): 967–974. doi:10.1161/CIRCRESAHA.110.220673.

Reactive Oxygen Species Originating from Mitochondria Regulate the Cardiac Sodium Channel

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Abstract

Rationale—Pyridine nucleotides regulate the cardiac Na^+ current (I_{Na}) through generation of reactive oxygen species (ROS).

Objective—We investigated the source of ROS induced by elevated NADH.

Methods and Results—In HEK cells stably expressing the cardiac Na^+ channel, the decrease of I_{Na} (52±9%; P<0.01) induced by cytosolic NADH application (100 μmol/L) was reversed by mitoTEMPO, rotenone, malonate, DIDS, PK11195 and 4′-chlorodiazepam, a specific scavenger of mitochondrial superoxide and inhibitors of the mitochondrial complex I, complex II, voltage-dependent anion channels, and benzodiazepine receptor, respectively. Antimycin A (20 μmol/L), a complex III inhibitor known to generate ROS, decreased I_{Na} (51±4%, P<0.01). This effect was blocked by NAD^+, forskolin, or rotenone. Inhibitors of complex IV, nitric oxide synthase, the NADPH oxidases, xanthine oxidases, the mitochondrial permeability transition pore, and the mitochondrial ATP-sensitive K^+ channel did not change the NADH effect on I_{Na}. Analogous results were observed in cardiomyocytes. Rotenone, mitoTEMPO, and 4′-chlorodiazepam also blocked the mutant A280V glycerol-3-phosphate dehydrogenase 1-like effect on reducing I_{Na}, indicating a role for mitochondria in the Brugada Syndrome caused by this mutation. Fluorescent microscopy confirmed mitochondrial ROS generation with elevated NADH and ROS inhibition by NAD^+.

Conclusions—Altering the oxidized to reduced NAD(H) balance can activate mitochondrial ROS production, leading to reduced I_{Na}. This signaling cascade may help explain the link between altered metabolism, conduction block, and arrhythmic risk.

Keywords

metabolism; pyridine nucleotides; arrhythmia; sudden death

Introduction

Recently, we reported that mutations in glycerol-3-phosphate dehydrogenase 1-like (GPD1-L) protein, a gene associated with Brugada Syndrome and Sudden Infant Death Syndromes, [1, 2] cause reduced cardiac sodium channel ($Na_v1.5$) function by modulating pyridine nucleotides.[3] Elevated intracellular NADH results in a rapid decrease in cardiac Na^+ current (I_{Na}) in cardiomyocytes that is large enough to be clinically significant[4] and of a magnitude

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Disclosures

Dr. Dudley has filed provisional patents related to this work: 1) Modulation of sodium current by nicotinamide adenine dinucleotide and 2) Modulating mitochondrial reactive oxygen species to increase cardiac sodium channel current and mitigate sudden death.

seen in Brugada Syndrome.[5] The effect is identical on heterologously expressed sodium channel in human embryonic kidney (HEK) cells. The immediacy of the NADH effect on reducing I_{Na} and the lack of change in mRNA abundance under various experimental conditions suggests that the effect of NADH is post-transcriptional.

NADH modulated $Na_v1.5$ through PKC activation and increased oxidative stress.[3] The finding that the balance of oxidized and reduced NAD(H) regulates I_{Na} suggests that the metabolic state of myocytes may influence $Na_v1.5$. NADH is known to oscillate with myocardial ischemia, and mitochondrial injury is associated with increased NADH and ROS levels.[6, 7] These changes in NADH could contribute to reduced I_{Na}, conduction block, and arrhythmic risk known to exist with ischemia. Moreover, heart failure is associated with increased oxidative stress, reduced NAD^+, and increased NADH.[8–10] The increased NADH level may contribute to the increased oxidative stress and diminished I_{Na} in heart failure.[11, 12]

Several metabolic pathways are known to produce ROS, including uncoupled nitric oxide synthase (NOS), the NAD(P)H oxidase, xanthine oxidase, and the mitochondrial electron transport chain (ETC). Cardiac oxidation leads to NOS uncoupling and diastolic dysfunction.[13] NAD(P)H oxidases are an important source of superoxide in human atherosclerosis.[14] Xanthine oxidase plays an important role in various forms of ischemic injury and in chronic heart failure.[15] In ischemia/reperfusion injury, the ETC serves as the source of ROS.[16] In chronic heart failure, ROS levels increase[17, 18] and myocardial antioxidant reserve decreases.[19, 20] In turn, ROS increases cell death by apoptosis, reduces cellular respiration, induces structural damage to proteins including ion channels, and impairs contractility.[8] Here, we show that mitochondria are the main source of NADH-dependent ROS downregulating the cardiac $Na_v1.5$.

Methods

Full descriptions of the methods are available in the supplemental material.

Cell Culture

We maintained a human embryonic kidney (HEK) cell line stably expressing the human cardiac $Na_v1.5$ channel (SCN5A cells). Expression of $Na_v1.5$ was linked to green fluorescent protein (GFP) expression by an internal ribosomal entry site (SCN5A-IRES-GFP). SCN5A cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum, 0.2 mg/mL geneticin (for antibiotic selection) and 1% penicillin/streptomycin in a 95% O_2/5% CO_2 incubator at 37°C. Rat neonatal ventricular myocytes (NVM) were isolated from neonatal rat hearts by collagenase treatment (Worthington Biochemical Corporation, Lakewook, NJ).

Nearly undetectable levels of GPD1-L protein are expressed in HEK cells.[1] Therefore, for whole-cell patch clamping experiments to study GPD1-L effects on $Na_v1.5$, SCN5A cells were transiently transfected with WT or A280V GPD1-L (a generous gift from Dr. Barry London, University of Pittsburgh, PA) and an expression vector containing red fluorescent protein (RFP) as described previously.[2] In these experiments, cells expressing both GFP and RFP were studied.

Electrophysiology

Na^+ currents were measured using the whole-cell patch clamp technique in voltage-clamp mode, while NVM action potentials (APs) were measured in current-clamp mode at room temperature. To measure Na^+ currents, pipettes (1–2 M) 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 130, CsCl 5, $CaCl_2$ 2, $MgCl_2$ 1.2, HEPES 10 and glucose 5 (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 $Na_v1.5$. Peak currents obtained during steps to −20 or −30 mV were used for comparison in determining the relative reduction of I_{Na}. To minimize time-dependent driftin gating parameters, all protocols were initiated 2–5 minafter whole-cell configuration was obtained. The currents were normalized for cell capacitance prior to deriving ratios.

For APs measurement, pipettes (2–4 MΩ) were filled with (in mmol/L): NaCl, 10, potassium glutamate 130, EGTA 1.0, $MgCl_2$ 0.5, KCl 9, HEPES 10, glucose 10, and MgATP 5 (adjusted to pH 7.4 with KOH). The bath solution consisted of (in mmol/L): NaCl 140, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1.0, HEPES 10, glucose 10 (adjusted to pH 7.4 with NaOH). APs were evoked by 4-ms current injections applied at 0.8–1 Hz. The upstroke velocity of the action potential was taken as the maximum values of dV/dt.

Inhibitors or activators were applied directly to the pipette solution except for apocynin, forskolin, NAD^+, and malonate, which were applied to bath solution. Concentrations were determined in our laboratory or by using values similar to those in the literatures.

Intracellular NADH Level

Intracellular NADH levels ($[NADH]_i$) were detected by using the EnzyChromTM NAD^+/NADH Assay Kit (BioAssay Systems, Hayward, CA) in SCN5A cells with or without treatment of 1 and 10 mmol/L pyruvate/lactate (PL) buffer for 10 min at room temperature. 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 $[NADH]_i$.

Confocal Microscopy

To measure mitochondrial ROS, the fluorecent probe MitoSOX™ Red was used according to the manufacturer's protocol. Briefly, three groups of SCN5A cells or rat NVM were studied: untreated cells, the PL group (cells treated with the PL buffer for 10 min to increase intracellular NADH levels[21–23], see "Results"), and the NAD-PL group (cells incubated with NAD^+ for ~6 hours at 37 °C and then treated with the PL buffer buffer for 10 min). Cells were first stained with Hoechst 33342 (0.4 μg/ml) and then incubated with 2.5 μmol/L MitoSOX™ Red for 10 min at 37 °C, followed by washing three times with warm Hank's balanced salt solution. Images were taken on a Zeiss LSM510 META confocal microscope (Carl Zeiss GmbH, Oberkochen, Germany) using an argon laser excitation (514 nm) with emisson collection through a 560 nm longpass filter. The cell area was calculated, and the whole cell fluorescence of MitoSOX™ Red was measured with ImageJ software. The number of pixels of the cell fluorescence divided by the cell area was used to determine the mitochondrial ROS generation.

To measure the effect of elevated intracellular NADH level on the mitochondrial membrane potential ($\Delta\Psi_m$), we applied the fluorescent probe tetramethylrhodamine methyl ester (TMRM), which is readily sequestered by mitochondria. SCN5A cells or rat NVM were loaded with TMRM (100 nmol/L)[24] for 30 min at 37 °C. Cells were washed three times with the bath solution used in the voltage-clamp experiments before being placed in a 35°C holder on the stage of the Zeiss confocal microscope. TMRM was excited with a helium neon laser at (543 nm), and the emission was collected through a 560 nm longpass filter. Images were collected in time series. Then cells were exposed to the mitochondrial uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP, 10 μmol/L) for 1 min at 35

°C with cells, which is sufficient to completely depolarize $\Delta\Psi_m$.[25] Images were collected in time series.

Statistical Evaluations

Data are shown as the mean ± SEM. 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

Sources of ROS Induced by NADH

Since SOD is able to block the effect of NADH,[3] ROS are implicated in the signaling cascade whereby NADH reduces I_{Na}. Sources of ROS within a cell include uncoupled NOS, the NAD(P)H oxidases, xanthine oxidase, and mitochondria. By using specific inhibitors, we tested which of these was the source of ROS modulating I_{Na} in response to increased cytosolic NADH.

Figure 1 shows that apocynin, N^{ω}-nitro-L-arginine methyl ester (L-NAME), and allopurinol did not affect I_{Na}, when they were applied alone in SCN5A cells. When applied with 100 μmol/L NADH, none of these blockers were able to inhibit the NADH effect on reducing cardiac I_{Na}. Steady state activation (SSA) was minimally affected by these compounds, and there were physiologically nonsignificant trends for hyperpolarizing shifts in steady state inactivation (SSI) with apocynin and allopurinol in the presence of NADH (Online Table I). These experiments indicate that the NAD(P)H oxidases, uncoupled NOS, and xanthine oxidases are not the source of ROS induced by NADH.

MitoTEMPO is a highly positively charged TEMPO derivative that is concentrated in the mitochondria matrix and acts there as a superoxide scavenger.[26, 27] MitoTEMPO at 5 μM blocked the NADH effect on reducing I_{Na} but had no effect on I_{Na} when applied alone (Fig. 1). The SSA and SSI were not affected by mitoTEMPO with or without the presence of NADH (Online Table I). This implied that the mitochondria were a likely source of ROS induced by increased NADH.

Mitochondrial ROS Generation Induced by Elevated NADH

Mitochondrial ROS generation was monitored with MitoSOX™ Red in SCN5A cells and rat NVM, respectively. MitoSOX™ Red is a membrane permeant, fluorogenic dye for selective detection of superoxide in the mitochondria. Once in the mitochondria, the dye is oxidized by superoxide and exhibits red fluorescence. Application of MitoSOX™ Red in untreated cells revealed a low level of red fluorescence, indicating low levels of mitochondrial ROS (Fig. 2). SCN5A cells and rat NVM were treated with 1 and 10 mmol/L PL buffer (PL group in Fig. 2). This PL buffer increased intracellular NADH level by 1.7±0.1-fold and decreased I_{Na} to 0.54±0.04 of control ($P<0.01$).[3] Treatments showed 2.06 ± 0.09-fold and 2.18 ± 0.15-fold increases in mitochondrial ROS levels for SCN5A cells and rat NVM as compared to untreated cells, respectively. This increase in ROS was blocked by NAD^+ pre-incubation (NAD-PL group in Fig. 2, 0.96 ± 0.06 and 1.11 ± 0.18-fold of untreated cells, respectively). These observations are in agreement with the electrophysiological studies and confirm that mitochondria are the source of ROS overproduction induced by elevated NADH.

The ETC as a Source of NADH-Induced ROS

Our previous work has shown that PKC activation is required for ROS production in response to NADH.[3] The ETC and mitochondrial ATP-sensitive K^+ channel (mitoK_{ATP}) are targets of PKC activation,[28] and both have been shown to be involved in ROS generation

and release from mitochondria.[6, 16, 29, 30] An inhibitor and an opener of the mitoK$_{ATP}$ channel, 5-hydroxydecanoate (5-HD)[31] and diazoxide[32] respectively, were applied to study whether they would have any effect on I_{Na}. As shown in Fig. 3A, 5-HD neither blocked the NADH effect on reducing I_{Na} nor showed any effect on I_{Na} when applied alone. Diazoxide did not affect I_{Na}, either. For 5-HD, there were minor shifts of $V_{1/2}$ values of the SSA and SSI relationships that were not enough to affect the evaluation of the peak currents (Online Table I). These experiments indicate that the mitoK$_{ATP}$ channel is not involved in NADH modulation of Na$_v$1.5.

Complexes I and III are the main sources of ROS production of ETC.[6, 16, 33] Rotenone, which decreases ROS generation by inhibiting complex I (i.e. NADH dehydrogenase),[6, 33] blocked entirely the NADH effect on I_{Na} (Fig. 3A), indicating that the ETC was the source of ROS overproduction induced by NADH. Malonate, an inhibitor complex II,[33] also blocked the NADH effect and reversed the decrease in I_{Na} (Fig. 3B). Fig. 3B also shows that azide, which inhibits complex IV,[33] failed to block NADH effect on reducing I_{Na}. Antimycin A blocks the electron transfer from the Q_i to Q_o sites of complex III and increases ROS generation in the intermembrane space of mitochondria.[6, 33] We found that antimycin A gave rise to an equivalent decrease of I_{Na} as did NADH. Comparably to NADH, the antimycin A effect was blocked by NAD^+, forskolin, or rotenone as shown in Fig. 3B.[3] A PKC inhibitor, chelerythrine, failed to block the antimycin A effect to reduce I_{Na}, as shown in Fig. 3C. This confirmed that PKC activation was necessary for ROS generation from complex III.[3] Shifts of $V_{1/2}$ values of SSI were observed with rotenone and azide in the presence of NADH, and with antimycin A alone. These were minor and unlikely to be sufficient to affect Na^+ channel availability significantly at the holding potential used (Online Table I).

NADH-Induced ROS Release from Mitochondria was through the Mitochondrial Inner Membrane Anion Channel (IMAC)

Mitochondrial respiration is ordinarily accompanied by low-level ROS generation. In the event of significant cellular ROS, mitochondria respond by increasing their own ROS production, a phenomenon termed ROS-induced ROS release (RIRR).[34, 35] Two modes of RIRR have been reported: the mitochondrial inner membrane anion channel (IMAC)-dependent and the mitochondrial permeability transition pore (MPTP)-dependent mechanisms. These two anions channels along with the voltage-dependent anion channel (VDAC) are thought to be the predominant paths for cytosolic release of superoxide generated by the ETC. Cycloporine A (CsA) and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) are inhibitors of MPTP and IMAC/VDAC, respectively. Fig. 3D shows that DIDS blocked the NADH effect on reducing I_{Na}, but CsA did not. Measurements of the mitochondrial $\Delta\Psi_m$ with TMRM showed that elevated NADH levels did not affect the $\Delta\Psi_m$ (data not shown). This indicated that the IMAC or VDAC but not MPTP are involved in ROS release in response to NADH.

IMAC is regulated by the mitochondrial benzodiazepine receptor (mBzR). It has been reported that ROS generation and oscillations are prevented by inhibiting IMAC with mBzR ligands such as 4′-chlorodiazepam (4′-CD) and PK11195.[6] Inhibition of mitochondria ROS release by 4′-CD is thought to prevent reperfusion arrhythmias.[24] As shown in Fig. 3D, both 4′-CD and PK11195 were capable of blocking the NADH effect on I_{Na}. Since the mBzR modifies ROS release through the IMAC, these data strenthen the idea that IMAC is involved in mitochondrial ROS release in response to NADH. FGIN-1-27 (500 μmol/L), an activator of mBzR[6, 24], showed that simply opening the mBzR was not enough to decrease I_{Na} (1.01 ± 0.14 of SCN5A group, P>0.05). When FGIN-1-27 and NADH were applied together, FGIN-1-27 showned no influence on the reduction in I_{Na} mediated by NADH. NADH (100 μmol/L) alone reduced I_{Na} to 0.54 ± 0.04 of SCN5A group (P<0.01)[3], while in

the presence of FGIN-1-27 (500 μmol/L), the reduction of I_{Na} by NADH was 0.51 ± 0.04 (P<0.01). This implies that the mBzR is fully activated in the presence of NADH.

Neonatal Ventricular Myocytes Show Similar Results

Analogous experiments were repeated using rat NVM to confirm the effects of rotenone, antimycin A, 4′-CD, and L-NAME on NADH regulation of $Na_v1.5$. As shown in Fig. 4, rotenone and 4′-CD blocked the NADH effect on I_{Na}, while L-NAME did not. Antimycin A reduced I_{Na} to 55 ± 7% in myocytes. These results were in agreement with the findings obtained with SCN5A cells, confirming the mitochondrial role on NADH regulation of $Na_v1.5$ in myocytes.

NADH treatment did not affect the maximum diastolic membrane potential. The value for untreated NVM was −66.9 ± 1.4 mV and was −64.3 ± 1.8 mV for myocytes treated with 500 μmol/L NADH (p=NS). On the other hand, treatment with NADH decreased the maximum upstroke velocity of the action potential to 0.68 ± 0.12 of untreated NVM (P<0.05).

A280V GPD1-L and NADH Affect I_{Na} Correspondingly

Previously, we have found that the mutant A280V GPD1-L reduces I_{Na} by increasing intracellular NADH.[3] Similarly to the NADH-mediated I_{Na} reduction, mitoTEMPO, rotenone, and 4′-CD all reversed the I_{Na} decrease caused by A280V GPD1-L (Fig. 5). When these compounds were applied to cells expressing WT GPD1-L, the I_{Na} was unvaried (data not shown). These results imply that increased NADH mediates the effect of A280V GPD1-L to downregulate $Na_v1.5$ and that mitochondrial ETC and IMAC are involved in the pro-arrhythmic effect of this mutation.

Discussion

Many signaling pathways involved in cardiomyopathy and cardioprotection converged on the mitochondria. Mitochondria comprise ~30–40% of the myocyte volume and generate >90% of the ATP.[36, 37] Also, they are a major site of physiological ROS production in the cardiomyocyte, with 1–3% of the electrons flowing through the ETC leaking to produce ROS.[38, 39] ROS generation within the mitochondrial matrix depends critically on the proton motive force, the $NADH/NAD^+$ ratio, the $CoQH_2/CoQ$ ratio, and the local O_2 concentration. Under conditions of a high $NADH/NAD^+$ ratio, complex I and perhaps other enzymes linked to the NADH pool may contribute to ROS production.[40]

In the present study, we found that the oxidative stress induced by NADH is derived from mitochondria. Experiments with different inhibitors for the uncoupled NOS, NAD(P)H oxidases, xanthine oxidases, mitoK_{ATP}, and the ETC revealed that the mitochondrial ETC plays a critical role in NADH regulation of $Na_v1.5$. Blockade of the NADH effect to reduce I_{Na} was observed with rotenone and malonate, complex I and II blockers, respectively. Because malonate inhibited the NADH-induced ROS but cannot prevent ROS release from complex I, it seemed likely that complex III was the source of ROS in our study. Another possibility is reverse electron transfer from complex II to complex I can also lead to ROS production.[41] This is also blocked by malonate and rotenone. Antimycin A inhibits complex III at the Q_i center and increases superoxide generation from the Q_o center.[42] In the present study, antimycin A caused a significantly reduced I_{Na}, supporting the idea that complex III is the source of ROS induced by NADH. At the same time, the antimycin A effect could be blocked by NAD^+, forskolin, and rotenone. These results are comparable to the inhibition of the NADH effect on I_{Na} reported in this and previous work.[3] Taken together, the data suggest that complex III is the main source of NADH-induced ROS generation and that

blockade of electron flow upstream of complex III minimizes ROS production induced by NADH.

ROS produced by leakage of electrons from the ETC can trigger the opening of the mitochondrial IMAC and subsequent release of $O_2^{\bullet-}$ to the cytoplasm.[6, 34] IMAC-dependent ROS release is regulated by the mBzR. Localized mitochondrial ROS release can propagate throughout cardiac cells in the form of oscillations or waves.[6, 34] Mitochondrial depolarization associated with increase ROS and activation of the MPTP has been correlated with opening of the mitoK_{ATP} channel and conduction block, referred to as a metabolic sink.[43] We show a second possible mechanism for conduction impairment involving mitochondrial ROS, ROS induced decreased I_{Na}, which is dependent on the mBzR and IMAC but not the MPTP. CsA failed to block the NADH effect on reducing I_{Na} while PK11195 and 4′-CD inhibited the NADH effect. This suggests that Na^+ channel-mediated changes in conduction may precede those of mitoK_{ATP}, since the mitoK_{ATP} effect requires mitochondrial MPTP activation and mitochondrial depolarization whereas the NADH effect requires less extreme mitochondrial ROS production.

Studies of metabolic stress in isolated cardiac cells reveal that energy-sensitive K^+ channels in the sarcolemmal membrane can be activated spontaneously in an oscillatory manner.[44] These K^+ current oscillations are closely associated with whole cell metabolic oscillations. Modulation of the cellular action potential by these metabolic oscillations could result in arrhythmias in the heart after ischemia-reperfusion. Mitochondria have been identified as the source of the oscillations. K^+ channel opening compounds like diazoxide and nicorandil have been found to protect heart cells from ischemic or oxidative stress through a mechanism that involves the opening of mitoK_{ATP} channel.[32] In our work, the blocker for mitoK_{ATP}, 5-HD, was unable to protect against the NADH-mediated reduction in I_{Na}, and an opener of mitoK_{ATP}, diazoxide, did not affect I_{Na}, either. These results indicate that the NADH effect is unique and independent of mitoK_{ATP}.

In summary, elevated intracellular NADH leads to mitochondrial ROS overproduction that results in downregulation of the cardiac Na^+ channel. Mitochondrial ROS overproduction is mainly derived from complex III of the electron transport chain, and ROS is probably released into the cytoplasm through the IMAC, which is regulated by the mBzR (Fig. 6). A similar mechanism likely explains the arrhythmia syndromes induced by mutant GPD1-L protein,[1, 3] since the mutant GPD1-L A280V leads to an increase of intracellular NADH level and mitoTEMPO, rotenone, and 4′-CD block the A280V GPD1-L effect to reduce I_{Na}. Valdivia et al.[45] presented a somewhat different possible signaling pathway to explain the reduction in I_{Na} with mutations of GPD1-L. Nevertheless, the two proposals share many elements, including elevated NADH and PKC activation being involved in the signaling cascade. In experiments not shown, the lack of effect on I_{Na} of raising intracellular dihydroxyacetone phosphate, which should increase glycerol-3-phosphate production by glycerol-3-phosphate dehydrogenase catalysis without raising NADH levels, suggests that NADH and not glycerol-3-phosphate is mediating the reduction in current.

Our experiments do not unequivocally establish a mechanism by which mitochondrial ROS reduce I_{Na}. ROS could be having a direct effect on the channel, cause the channel to be excluded from the membrane, or alter channel post-translational modifications known to decrease I_{Na}. Preliminary experiments suggest that the disulfide reducing agent, dithiothreitol, does not prevent the NADH effect. Moreover, preliminary total internal reflection fluoroscopy experiments with labeled sodium channels do not show any channel internalization in response to NADH. It seems reasonable that PKC acts directly on the channel, as proposed by Valdivia et al[45]. Changes in the SSA and SSI relationships support this assertion. It is interesting to note, however, that the effect of only one of two GPD1-L

mutations known to cause sudden death is fully reversed by eliminating a Na^+ channel PKC phosphorylation site, suggesting the possibility of multiple mechanisms or sites being involved in the current reduction. Our results represent a heretofore unknown regulation of the cardiac Na^+ channel by NADH through mitochondria ROS production that may help explain the link between altered metabolism and arrhythmic risk.

Novelty and Significance

What Is Known?

- Cardiac arrhythmias are more prevalent when cardiac metabolism is abnormal.
- A mutation in glycerol-3-phosphate dehydrogenase like-1 protein (GPDL-1) alters pyridine nucleotide levels and reduces cardiac sodium current (I_{Na}), potentially explaining how this mutation leads to the Brugada Syndrome, which increases the likelihood of sudden cardiac death.

What New Information Does This Article Contribute?

- Elevation in NADH results in activation of protein kinase C (PKC) and a subsequent increase in mitochondrial complex III-derived reactive oxygen species (ROS) through ROS-induced ROS release involving the mitochondrial inner membrane anion channel (IMAC).
- Mitochondrial superoxide release is responsible for the downregulation of I_{Na}.
- Inhibition of mitochondrial ROS overproduction by several strategies prevents I_{Na} downregulation by NADH.

Altered cardiac metabolism is associated with increased risk of arrhythmias and sudden death. In part, this occurs because of reduced electrical conduction in the cardiomyocytes, but the mechanisms for this are not clear. We have shown previously that a mutation in GPD1-L protein, causing the sudden death condition Brugada syndrome, reduces I_{Na} by raising intracellular NADH levels and inducing ROS. Here, we investigated the source of ROS induced by elevated NADH. We found that elevated NADH induced ROS production from mitochondria and that ROS release from the mitochondria was mediated by the IMAC. NAD^+, inhibition of mitochondrial electron transport, a mitochondrial targeted antioxidant, and an IMAC modulator could prevent the reduction in I_{Na} by reducing mitochondrial ROS production. These findings contribute to our understanding of the mechanisms of conduction block and arrhythmia when cardiac metabolism is dysfunctional. Also, the results suggest possible therapeutic strategies to reduce arrhythmic risk associated with cardiomyopathy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Sergey Dikalov (Emory University, Atlanta, GA) for the gift of mitoTEMPO.

Sources of funding

This work was supported by NIH R01 HL085558, R01 HL073753, and P01 HL058000 (SCD).

Non-standard Abbreviations and Acronyms

4′-CD	4′-chlorodiazepam
5-HD	5 –hydroxydecanoate
AP	action potential
CCCP	carbonyl cyanide 3-chlorophenylhydrazone
CsA	Cycloporine A
DIDS	4′-diisothiocyanatostilbene-2,2′-disulfonic acid
ETC	electron transport chain
FGIN-1-27	[N,N-dihexyl-2-(4-fluorophenyl)indole-3-acetamide
GFP	green fluorescent protein
GPD1-L	glycerol-3-phosphate dehydrogenase 1-like
HEK	human embryonic kidney
IMAC	the mitochondrial inner membrane anion channel
L-NAME	N^{ω}-nitro-L-arginine methyl ester
mBzR	the mitochondrial benzodiazepine receptor
mitoK$_{ATP}$	mitochondrial ATP-sensitive K^+ channel
MPTP	the mitochondrial permeability transition pore
Na$_v$1.5	cardiac sodium channel
NOS	nitric oxide synthase
NVM	neonatal ventricular myocyte
PK	protein kinase
PL	pyruvate/lactate
RFP	red fluorescent protein
RIRR	ROS-induced ROS release
SCN5A	cardiac sodium channel
SOD	superoxide dismutase
SSA	steady state activation
SSI	steady state inactivation
TMRM	tetramethylrhodamine methyl ester
VDAC	the voltage-dependent anion channel

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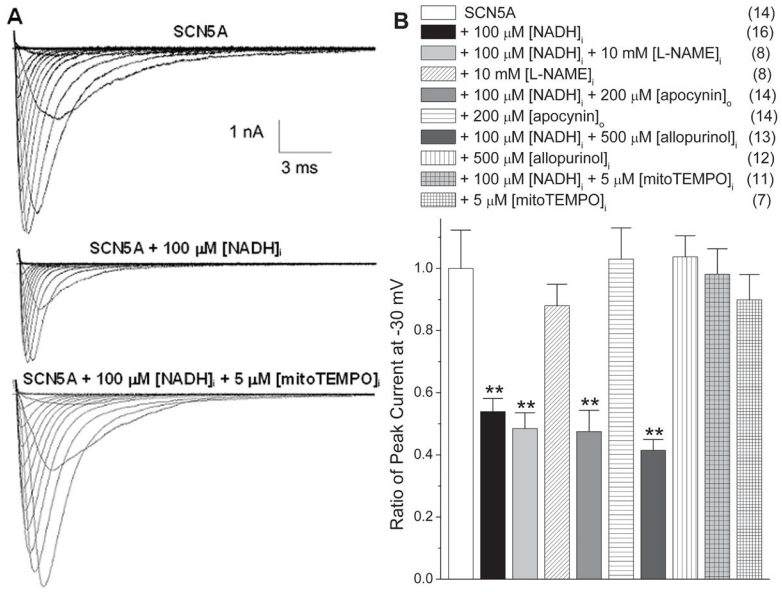

Figure 1.

The source of ROS induced by NADH is the mitochondria. (A) Representative traces of I_{Na} demonstrate the decrease in current in the presence of $[NADH]_i$ (100 μmol/L) was blocked by mitoTEMPO (5 μmol/L). (B) The downregulation of peak I_{Na} by $[NADH]_i$ at 100 μmol/L (**P<0.01 versus SCN5A group) is not reversed by L-NAME, apocynin, or allopurinol (P>0.05 versus NADH group), but is reversed by mitoTEMPO at 5 μmol/L (P>0.05 versus SCN5A group, P<0.01 versus NADH group). All these compounds have no effect on I_{Na} when applied alone (P>0.05 versus SCN5A group). Numbers in parentheses indicate the number of experiments.

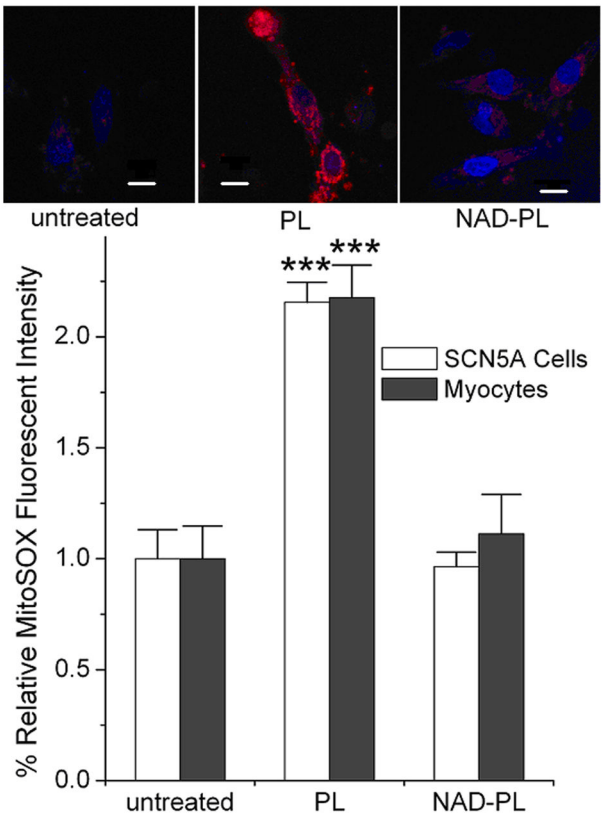

Figure 2.

Mitochondrial ROS production in response to $[NADH]_i$ was monitored by MitoSOX™ Red with SCN5A cells and myocytes. The control groups were untreated, the PL groups were treated with 1 and 10 mmol/L pyruvate/lactate for 10 min, and the NAD-PL groups were incubated with 500 μmol/L NAD^+ for ~6 hours and then treated with pyruvate/lactate buffer for 10 min. The pictures in the upper panel are representative images of myocytes of three groups. The scale bar indicates 10 μm. The lower panel shows the relative MitoSOX™ Red fluorescent intensity, ***P<0.001 versus the untreated cells or NAD-PL groups. For each group, 9–16 samples were averaged.

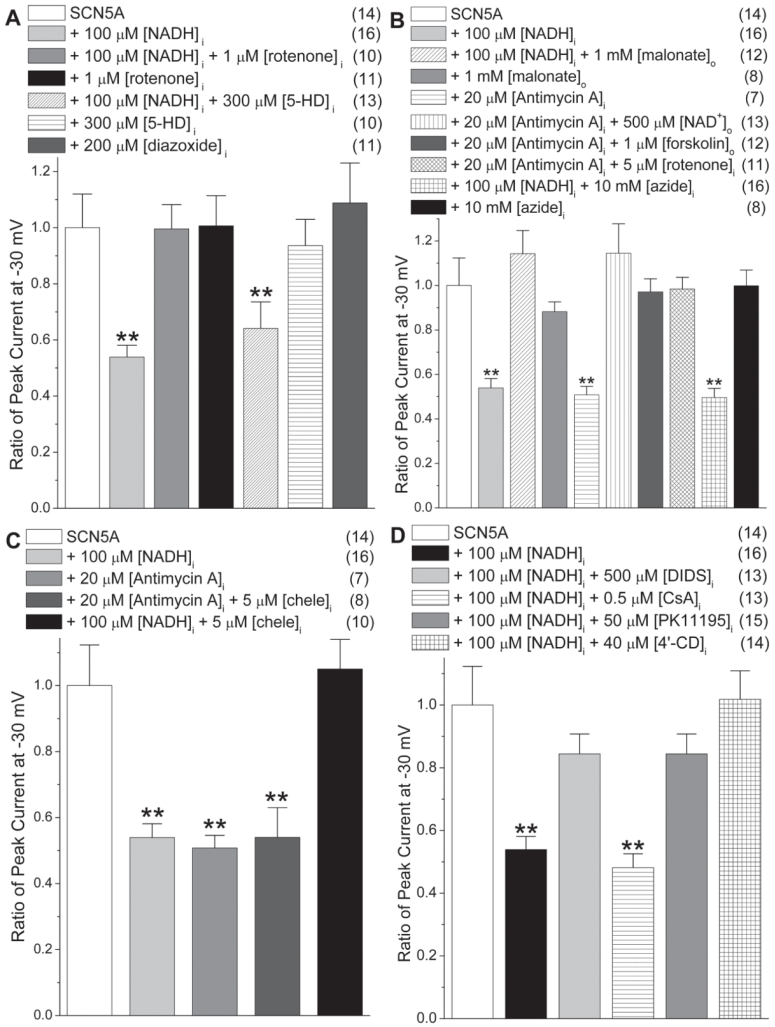

Figure 3.

PKC, the electron transport chain and the IMAC are involved in downregulation of I_{Na} by $[NADH]_i$. (A) Downregulation of I_{Na} by $[NADH]_i$ (**P<0.01 versus SCN5A) is reversed by rotenone (1 μmol/L), but not by 5-HD. Diazoxide does not affect I_{Na} (P>0.05 versus SCN5A). (B) Malonate (1 mmol/L) blocks the NADH effect on reducing I_{Na}, and antimycin A (20 μmol/L) reproduces the $[NADH]_i$ effect (**P<0.01 versus SCN5A group). The antimycin A-induced reduction in I_{Na} is prevented by $[NAD^+]_o$, forskolin, or rotenone. Azide failed to block the NADH effect. (C) Chelerythrine failed to block the antimycin A effect on reducing I_{Na}, confirming that PKC activation is required for ROS generation. (D) Downregulation of I_{Na} by $[NADH]_i$ is reversed by DIDS, PK11195 and 4′-CD, but not by CsA (**P<0.01 versus SCN5A groups). Numbers in parentheses indicate the number of experiments.

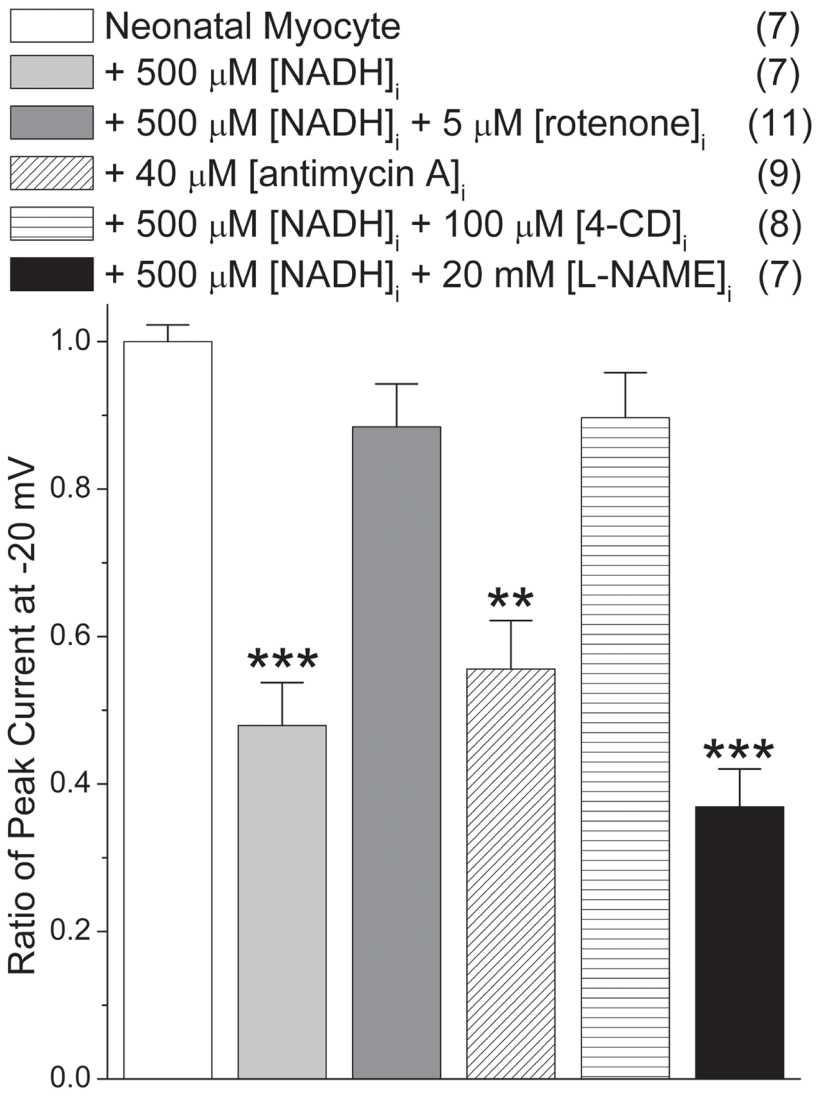

Figure 4.

Neonatal ventricular myocytes show analogous downregulation of I_{Na} by $[NADH]_i$. Downregulation can be blocked by rotenone and 4′-CD, but not L-NAME. Antimycin A decreases I_{Na} similarly to that of $[NADH]_i$ (**P<0.01 and ***P<0.001 versus control myocytes). Numbers in parentheses indicate the number of experiments.

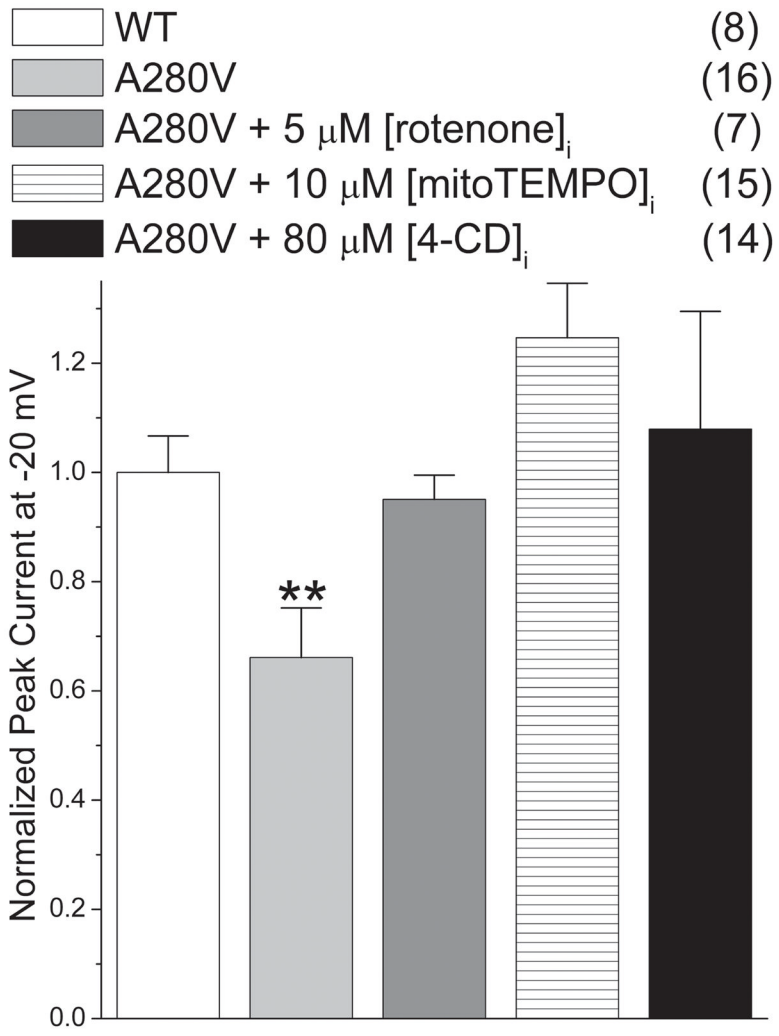

Figure 5.

Downregulation of I_{Na} by A280V GPD1-L is reversed by mitoTEMPO, rotenone, and 4′-CD (**P<0.01 versus all other groups).). Peak currents at −20 mV were normalized to cell capacitance and divided by the current obtained with SCN5A cells transfected with WT GPD1-L. Numbers in parentheses indicate the number of experiments.

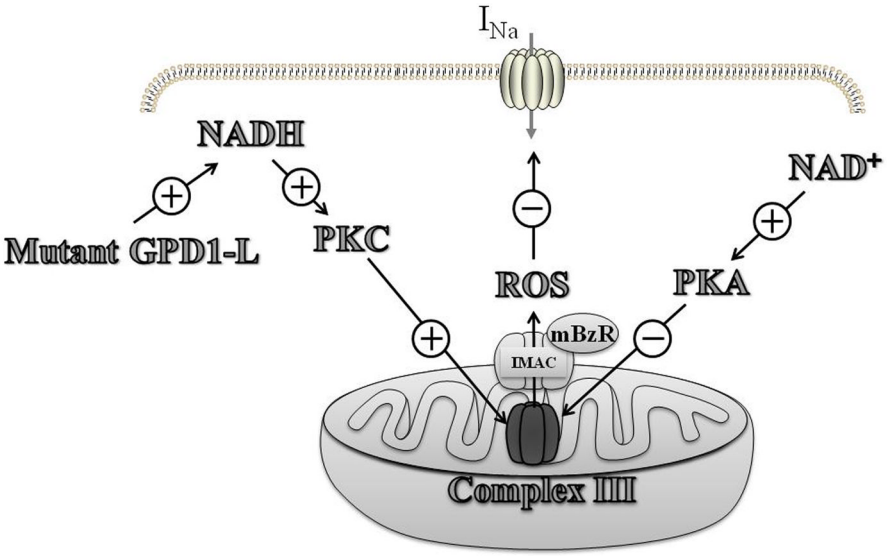

Figure 6.

A proposed signaling pathway by which the mutant GPD1-L and NADH downregulate cardiac Na^+ channel by causing PKC activation and ROS overproduction from the complex III of mitochondrial electron transport chain. Reactive oxygen species (ROS) are released from the mitochondria by the IMAC that is modulated by the mBzR. NAD^+ upregulates the cardiac Na^+ channel through PKA activation and inhibition of ROS overproduction.