Reactive Oxygen Species Originating From Mitochondria Regulate the Cardiac Sodium Channel

Man Liu, Hong Liu, Samuel C. Dudley, Jr

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 human embryonic kidney (HEK) cells stably expressing the cardiac Na\(^+\) channel, the decrease of \(I_{Na}\) (52\(\pm\)9\%; \(P<0.01\)) induced by cytosolic NADH application (100 \(\mu\)mol/L) was reversed by mitoTEMPO, rotenone, malonate, DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid), 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. Anti-mycin A (20 \(\mu\)mol/L), a complex III inhibitor known to generate ROS, decreased \(I_{Na}\) (51\(\pm\)4\%, \(P<0.01\)). This effect was blocked by NAD\(^+\), forskolin, or rotenone. Inhibitors of complex IV, nitric oxide synthase, the NAD(P)H 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 GPD1-L (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. (Circ Res. 2010;107:967-974.)

Key Words: metabolism ■ pyridine nucleotides ■ arrhythmia ■ sudden death

Recently, we reported that mutations in GPD1-L (glycerol-3-phosphate dehydrogenase 1-like) protein, a gene associated with Brugada syndrome and sudden infant death syndromes,\(^{1,2}\) cause reduced cardiac sodium channel (Na\(_{1.5}\)) 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 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 posttranscriptional.

NADH modulates Na\(_{1.5}\) through protein kinase (PK)C 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\(_{1.5}\). 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\(^+\),\(^{8}\) 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. Here, we show that mitochondria are the main source of NADH-dependent ROS downregulating the cardiac Na1.5.

**Methods**

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Cell Culture**

We maintained a HEK cell line stably expressing the human cardiac Na1.5 channel (SCN5A cells). Expression of Na1.5 was linked to ion channels, and impairs contractility. Here, we show that mitochondria are the main source of NADH-dependent ROS downregulating the cardiac Na1.5.

Near undetectable levels of GPD1-L protein are expressed in HEK cells. Therefore, for whole-cell patch clamping experiments to study GPD1-L effects on Na1.5, SCN5A cells were transiently transfected with wild-type or A280V GPD1-L (a generous gift from Dr Barry London, University of Pittsburgh, Pa) and an expression vector containing red fluorescent protein as previously described. In these experiments, expression both GFP and red fluorescent protein were studied.

**Electrophysiology**

Na+ currents were measured using the whole-cell patch clamp technique in voltage-clamp mode, whereas NVM action potentials (APs) were measured in current-clamp mode at room temperature. To measure Na+ currents, pipettes (1 to 2 MΩ) were filled with a pipette solution containing (in mmol/L): CsCl 80, cesium aspartate 80, EGTA 11, MgCl2 1, CaCl2 1, HEPES 10, and Na2ATP 5 (adjusted to pH 7.4 with CsOH). The bath solution consisted of (in mmol/L): NaCl 130, CsCl 5, CaCl2 2, MgCl2 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 Na1.5. Peak currents obtained during steps to −20 or −30 mV were used for comparison in determining the relative reduction of INa, To minimize current-dependent drift in gating parameters, all protocols were initiated 2 to 5 minutes after whole-cell configuration was obtained. The currents were normalized for cell capacitance before deriving ratios.

For APs measurement, pipettes (2 to 4 MΩ) were filled with (in mmol/L): NaCl 10, potassium glutamate 130, EGTA 1.0, MgCl2 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, CaCl2 2, MgCl2 1.0, HEPES 10, glucose 10 (adjusted to pH 7.4 with NaOH). APs were evoked by 4-msec current injections applied at 0.8 to 1 Hz. The upstroke velocity of the AP 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 the bath solution. Concentrations were determined in our laboratory or by using values similar to those in the literature.

**Intracellular NADH Level**

Intracellular NADH levels ([NADH]) were detected by using the EnzyChrom NAD+/NADH Assay Kit (BioAssay Systems, Hayward, Calif) in SCN5A cells with or without treatment of 4,4′-DIDS. 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 (ΔΨm), we applied the fluorescent probe tetramethylrhodamine methyl ester (TMRM), which is readily sequestered by mitochondria. SCN5A cells or rat NVMs were loaded with TMRM (100 nmol/L) for 30 minutes at 37°C. Cells were washed 3 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 emission collection through a 560-nm long-pass 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.

**Confocal Microscopy**

To measure mitochondrial ROS, the fluorescent probe MitoSOX Red was used according to the protocol of the manufacturer. Briefly, 3 groups of SCN5A cells or rat NVMs were studied: untreated cells, the PL group (cells treated with the PL buffer for 10 minutes to increase intracellular NADH levels, see Results), and the NAD-PL group (cells incubated with NAD+ for 6 hours at 37°C and then treated with the PL buffer for 10 minutes). Cells were first stained with Hoechst 33342 (0.4 μg/mL) and then incubated with 2.5 μmol/L MitoSOX Red for 10 minutes at 37°C, followed by washing 3 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 emission collection through a 560-nm long-pass 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 (ΔΨm), we applied the fluorescent probe tetramethylrhodamine methyl ester (TMRM), which is readily sequestered by mitochondria. SCN5A cells or rat NVMs were loaded with TMRM (100 nmol/L) for 30 minutes at 37°C. Cells were washed 3 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 long-pass filter. Images were collected in time series. Then, cells were exposed to the mitochondrial uncoupler carbonyl cyanide 3-chlorophenylhydrazone (10 μmol/L) for 1 minute at 35°C with cells, which is sufficient to completely depolarize ΔΨm. Images were collected in time series.
Statistical Evaluations
Data are shown as the means±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
Because superoxide dismutase is able to block the effect of NADH,3 ROS are implicated in the signaling cascade whereby NADH reduces $I_{\text{Na}}$. 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 $I_{\text{Na}}$ in response to increased cytosolic NADH.

Figure 1 shows that apocynin, Nω-nitro-L-arginine methyl ester (L-NAME), and allopurinol did not affect $I_{\text{Na}}$ when they were applied alone in SCN5A cells. When applied with 100 μmol/L NADH, none of these blockers was able to inhibit the NADH effect on reducing cardiac $I_{\text{Na}}$. 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 μmol/L blocked the NADH effect on reducing $I_{\text{Na}}$ but had no effect on $I_{\text{Na}}$ when applied alone (Figure 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 NVMs, 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 (Figure 2). SC5N5A cells and rat NVMs were treated with 1 and 10 mmol/L PL buffer (PL group in Figure 2). This PL buffer increased intracellular NADH level by 1.7±0.1-fold and decreased $I_{\text{Na}}$ 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 NVMs as compared with untreated cells, respectively. This increase in ROS was blocked by NAD$^+$ preincubation (NAD-PL group in Figure 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.

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$_{\text{ATP}}$) are targets of PKC activation,28 and both have been shown to be involved in ROS generation and release from mitochondria.5,16,29,30 An inhibitor and an opener of the mitoK$_{\text{ATP}}$ channel, 5-hydroxydecanoate (5-HD)31 and diazoxide,32 respectively, were applied to study whether they would have any effect on $I_{\text{Na}}$. As shown in Figure 3A, 5-HD neither blocked the NADH effect on reducing $I_{\text{Na}}$ nor showed any effect on $I_{\text{Na}}$ when applied alone. Diazoxide

Figure 1. The source of ROS induced by NADH is the mitochondria. A, Representative traces of $I_{\text{Na}}$ demonstrate the decrease in current in the presence of [NADH] (100 μmol/L) was blocked by MitoTEMPO (5 μmol/L). B, Downregulation of peak $I_{\text{Na}}$ by [NADH] at 100 μmol/L ($**P<0.01$ vs SCN5A group) is not reversed by L-NAME, apocynin, or allopurinol ($P>0.05$ vs NADH group) but is reversed by MitoTEMPO at 5 μmol/L ($P>0.05$ vs SCN5A group, $P<0.01$ vs NADH group). All of these compounds have no effect on $I_{\text{Na}}$ when applied alone ($P>0.05$ vs SCN5A group). Numbers in parentheses indicate the number of experiments.
NADH-Induced ROS Release From Mitochondria Was Through the Mitochondrial Inner Membrane Anion Channel

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). Two modes of ROS-induced ROS release have been reported: the mitochondrial inner membrane anion channel (IMAC)-dependent and the mitochondrial permeability transition pore (MPTP)-dependent mechanisms. These 2 anions channels, along with the voltage-dependent anion channel (VDAC), are thought to be the predominant paths for cytotoxic release of superoxide generated by the ETC. Cyclosporine A and DIDS (4,4′-disothiocyanatostilbene-2,2′-disulfonic acid) are inhibitors of MPTP and IMAC/VDAC, respectively. Figure 3D shows that DIDS blocked the NADH effect on reducing $I_{\text{Na}}$, but cyclosporine A did not. Measurements of the mitochondrial $\Delta W_{\text{m}}$ with TMRM showed that elevated NADH levels did not affect the $\Delta W_{\text{m}}$ (data not shown). This indicated that the IMAC or VDAC, but not MPTP, is 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. Inhibition of mitochondria ROS release by 4′-CD is thought to prevent reperfusion arrhythmias. As shown in Figure 3D, both 4′-CD and PK11195 were capable of blocking the NADH effect on $I_{\text{Na}}$. Because the mBzR modifies ROS release through the IMAC, these data strengthen the idea that IMAC is involved in mitochondrial ROS release in response to NADH. N,N-Dihexyl-2-(4-fluorophenyl)indole-3-acetamide (FGIN-1-27) (500 μmol/L), an activator of mBzR, showed that simply opening the mBzR was not enough to decrease $I_{\text{Na}}$ (1.01±0.14 of SCN5A group; P<0.05). When FGIN-1-27 and NADH were applied together, FGIN-1-27 showed no influence on the reduction in $I_{\text{Na}}$ mediated by NADH. NADH (100 μmol/L) alone reduced $I_{\text{Na}}$ to 0.54±0.04 of SCN5A group (P<0.01), whereas in the presence of FGIN-1-27 (500 μmol/L), the reduction of $I_{\text{Na}}$ 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 NVMs to confirm the effects of rotenone, anti–mycin A, 4′-CD, and L-NAME on NADH regulation of $I_{\text{Na}}$. As shown in Figure 4, rotenone and 4′-CD blocked the NADH effect on $I_{\text{Na}}$, whereas L-NAME did not. Anti–mycin A reduced $I_{\text{Na}}$ 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 $I_{\text{Na}}$ in myocytes.

NADH treatment did not affect the maximum diastolic membrane potential. The value for untreated NVMs 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 AP to 0.68 ± 0.12 of untreated NVMs (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. Similarly to the NADH-mediated $I_{Na}$ reduction, mitoTEMPO, rotenone, and 4′-CD all reversed the $I_{Na}$ decrease caused by A280V GPD1-L (Figure 5). When these compounds were applied to cells expressing wild-type 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 $I_{Na}$ and that mitochondrial ETC and IMAC are involved in the proarrhythmic effect of this mutation.

**Discussion**

Many signaling pathways involved in cardiomyopathy and cardioprotection converged on the mitochondria. Mitochondria comprise ≈30% to 40% of the myocyte volume and generate >90% of the ATP. Also, they are a major site of physiological ROS production in the cardiomyocyte, with 1% to 3% of the electrons flowing through the ETC leaking to produce ROS. 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.

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 $I_{Na}$. 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 could not prevent ROS release from complex I, it seemed likely that complex III was
Another possibility is reverse electron transfer from complex II to complex I can also lead to ROS production. This is also blocked by malonate and rotenone. Anti–mycin A inhibits complex III at the Q center and increases superoxide generation from the Qo center. In the present study, anti–mycin 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 anti–mycin A effect could be blocked by NADH, forskolin, and rotenone. These results are comparable to the inhibition of the NADH effect on $I_{Na}$ reported in this and previous work. 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 $O_2^-$ to the cytoplasm. 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. Mitochondrial depolarization associated with increased ROS and activation of the MPTP has been correlated with opening of the sarcolemmal KATP channel and conduction block, referred to as a metabolic sink. 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. Cyclosporine A failed to block the NADH effect on $I_{Na}$, whereas PK11195 and 4′-CD inhibited the NADH effect. This suggests that Na$^+$ channel-mediated changes in conduction may precede those of the KATP channel, because the KATP channel 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. These K$^+$ current oscillations are closely associated with whole-cell metabolic oscillations. Modulation of the cellular AP 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 mitoKATP channel. In our work, the blocker for mitoKATP, 5-HD, was unable to protect against the NADH-mediated reduction in $I_{Na}$, and an opener of mitoKATP, diazoxide, did not affect $I_{Na}$, either. These results indicate that the NADH effect is unique and independent of mitoKATP. 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 ETC, and ROS is probably released into the cytoplasm through the IMAC, which is regulated by the mBzR (Figure 6). A similar mechanism likely explains the arrhythmia syndromes induced by mutant GPD1-L protein, because 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 presented a somewhat
different possible signaling pathway to explain the reduction in \( I_{\text{Na}} \) with mutations of GPD1-L. Nevertheless, the 2 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 \( I_{\text{Na}} \) 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 \( I_{\text{Na}} \). ROS could be having a direct effect on the channel, cause the channel to be excluded from the membrane, or alter channel posttranslational modifications known to decrease \( I_{\text{Na}} \). 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. Changes in the SSA and SSI relationships support this assertion. It is interesting to note, however, that the effect of only 1 of 2 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.

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Disclosures
S.C.D. 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.

References
Mitochondrial superoxide release is responsible for the downregulation of $I_{\text{Na}}$.

- Inhibition of mitochondrial ROS overproduction by several strategies prevents $I_{\text{Na}}$ downregulation by NADH.

**Novelty and Significance**

**What Is Known?**

- Cardiac arrhythmias are more prevalent when cardiac metabolism is abnormal.
- A mutation in GPD1-L (glycerol-3-phosphate dehydrogenase 1-like) protein alters pyridine nucleotide levels and reduces cardiac sodium current ($I_{\text{Na}}$), 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 (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 $I_{\text{Na}}$.
- Inhibition of mitochondrial ROS overproduction by several strategies prevents $I_{\text{Na}}$ downregulation by NADH.

Altered cardiac metabolism is associated with increased risk of arrhythmias and sudden death. In part, this occurs because of reduced electric 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 $I_{\text{Na}}$ 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. NADH inhibition of mitochondrial electron transport, a mitochondrial targeted antioxidant, and an IMAC modulator could prevent the reduction in $I_{\text{Na}}$ 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.
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Reactive Oxygen Species Originating from Mitochondria Regulate the Cardiac Sodium Channel

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Methods

All chemicals were purchased from Sigma (St. Louis, MO) except: diazoxide and FGIN-1-27 (Enzo Life Sciences International, Inc., Plymouth Meeting, PA); chelerythrine and cyclosporin A (Alomone Labs, Jerusalem, Israel); and Hoechst 33342, MitoSOX™ Red, and tetramethylrhodamine methyl ester (TMRM) (Molecular Probes, Eugene, OR). MitoTEMPO was a generous gift from Dr. Sergey Dikalov (Emory University, Atlanta, GA).

Cell Culture

We maintained a human embryonic kidney (HEK) cell line stably expressing the human cardiac Na,1.5 channel (SCN5A cells). Expression of Na,1.5 was linked to green fluorescent protein (GFP) expression by an internal ribosom al 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% O₂/5% CO₂ 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. Therefore, for whole-cell patch clamping experiments to study GPD1-L effects on Na,1.5, 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. 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 at room temperature. Pipettes (1-2 MΩ) were filled with a pipette solution containing (in mmol/L): CsCl 80, cesium aspartate 80, EGTA 11, MgCl₂ 1, CaCl₂ 1, HEPES 10, and Na₂ATP 5 (adjusted to pH 7.4 with CsOH). The bath solution consisted of (in mmol/L): NaCl 130, CsCl 5, CaCl₂ 2, MgCl₂ 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 voltage-gated Na,1.5 channels. Peak currents obtained during steps to -20 or -30 mV were used for comparison in determining the relative reduction of \( I_{Na} \). Steady state fast inactivation was assessed during voltage depolarization from a holding potential of −140 to −20 mV for 500 ms, and measuring current at -20 mV. In all recordings, 80% of the series resistance was compensated, yielding a maximum voltage error of ~1 mV. Data were sampled at 50 kHz and later low pass filtered at 10 kHz for analysis. Currents were recorded and analyzed with an Axopatch 200B amplifier, Axon Digidata 1320A A/D converter and pClamp software (Molecular Devices, Sunnyvale, CA). To minimize time-dependent drift in gating parameters, all protocols were initiated 2-5 min after whole-cell configuration was obtained. The currents were normalized with cell capacitance prior to deriving ratios.

Rat NVM action potentials were measured using the whole-cell patch clamp technique in current-clamp mode at room temperature. Pipettes (2-4 MΩ) were filled with a pipette solution containing (in mmol/L): NaCl 10, potassium glutamate 130, EGTA 1.0, MgCl₂ 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, CaCl₂ 2, MgCl₂ 1.0, HEPES 10 and glucose 10 (adjusted to pH 7.4 with NaOH). Action potentials were evoked by brief (4 ms) current injections applied at 0.8-1 Hz. Eighty percent of the series resistance was compensated, yielding a maximum voltage error of ~1 mV. Data were sampled at 50 kHz and later low pass filtered at 10 kHz for analysis. Action potentials were recorded and analyzed with an Axopatch 200B amplifier, Axon Digidata 1320A A/D converter and pClamp software.

The following specific inhibitors or activators were applied directly in the pipette solution, alone or together: NADH (100-500 μmol/L), Nω-nitro-L-arginine methyl ester (L-NAME, 1-20 mmol/L), allopurinol (200 μmol/L), mitoTEMPO (5-20 μmol/L), rotenone (1-5 μmol/L), antimycin A (20-40 μmol/L), azide (10 mmol/L), 5-hydroxydecanoate (5-HD, 300 μmol/L), 4,4’-disothiocyanatostilbene-2,2’-disulfonic acid (DIDS, 500 μmol/L), cycloporine A (CsA, 0.5 μmol/L), PK11195 (50 μmol/L), 4’-chlorodiazepam (4’-CD, 40-100 μmol/L), and FGIN-1-27 (500 μmol/L). Apocynin (100-300 μmol/L),
forskolin (1-5 μmol/L), NAD⁺ (500 μmol/L) and malonate (1 mmol/L) were applied to bath solution, respectively. Concentrations were determined in our laboratory or by using the similar values used in literatures.

**Intracellular NADH Level**

Intracellular NADH levels ([NADH]ᵢ) were detected by using the EnzyChrom™ NAD⁺/NADH Assay Kit (BioAssay Systems, Hayward, CA) in SCN5A cells with or without treatment of 1 mmol/L pyruvate and 10 mmol/L lactate 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].

**Confocal Microscopy**

To measure mitochondrial ROS, the fluorescent 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 1 mmol/L pyruvate and 10 mmol/L lactate for 10 min at room temperature, under which condition intracellular NADH level was increased 3-5, see “Results”), and the NAD-PL group (cells incubated with NAD⁺ for ~6 hours at 37 °C and then treated with 1 mmol/L pyruvate and 10 mmol/L lactate for 10 min at room temperature). The three groups of cells were then incubated with 2.5 μM MitoSOX™ Red in Hank’s balanced salt solution (HBSS) for 10 min at 37 °C, followed by three times wash with warm HBSS. Before treatment with MitoSOX™ Red, cells were first stained with Hoechst 33342 (0.4 µg/ml working concentration) for 20 min at 37 °C. Images were taken on a Zeiss LSM510 META confocal microscope (Carl Zeiss GmbH, Oberkochen, Germany) using an argon laser excitation (514 nm) with emission collection at more than 560 nm (red). The cell area was calculated, and the whole cell fluorescence of MitoSOX™ Red was measured with ImageJ software. The number of pixels of cell fluorescence divided by the cell area was used to determine the mitochondrial ROS generation. For each of the groups, 9 to 16 cells were used. NADH in water has an emission peak at 460 nm and less than 20% of the maximum value above 560 nm. Therefore, NADH is unlikely to interfere with the fluorescence of MitoSOX™ Red in our experiments.

To measure the effect of elevated intracellular NADH level on the mitochondrial membrane potential (ΔΨₘ), the fluorescent membrane-permeant cationic probe TMRM, which is readily sequestered by mitochondria, was applied. SCN5A cells or rat NVM were loaded with TMRM (100 nmol/L) for 30 min at 37 °C in the dark. Then, cells were washed gently twice and kept with the bath solution used in the patch experiments before being placed on the stage of a Zeiss LSM510 META confocal microscope (35°C). TMRM was excited at 543 nm with a helium neon laser (3%), and the emission was collected through a 560 nm longpass filter. Images were collected at 30 s intervals for 10 min and then 2 min interval for 30 min. For the PL group, pyruvate and lactate were applied after the first image was taken. The mitochondrial uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP, 10 μmol/L) was incubated for 1 min at 35 °C with cells, which is sufficient to completely depolarize ΔΨₘ. Images were then collected every 2 min for 20 min. The resulting fluorescence images were processed using Zeiss LSM510 META software to obtain the time course of the TMRM fluorescence changes.

**Statistical Evaluations**

Data are shown as the mean ± SEM. Determinations of statistical significance were performed with ANOVA with Bonferroni correction for comparisons of multiple means. A value of P<0.05 was considered statistically significant.
### ONLINE TABLE I

**Online Table I. Parameters of voltage dependence of steady state activation and inactivation of all groups.**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Voltage dependence of activation</th>
<th>Voltage dependence of inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{1/2}$, mV</td>
<td>$k$, mV</td>
</tr>
<tr>
<td>SCN5A</td>
<td>-44.7 ± 0.2</td>
<td>5.7 ± 0.1</td>
</tr>
<tr>
<td>+ 100 µM [NADH]</td>
<td>-44.5 ± 0.3</td>
<td>5.5 ± 0.2</td>
</tr>
<tr>
<td>+ 100 µM [NADH] + 10 mM [L-NAME]</td>
<td>-45.9 ± 0.6</td>
<td>6.0 ± 0.5</td>
</tr>
<tr>
<td>+ 100 µM [NADH] + 200 µM [apocynin]</td>
<td>-43.2 ± 0.3</td>
<td>6.9 ± 0.2</td>
</tr>
<tr>
<td>+ 100 µM [NADH] + 200 µM [allopurinol]</td>
<td>-44.1 ± 1.1</td>
<td>6.6 ± 0.8</td>
</tr>
<tr>
<td>+ 100 µM [NADH] + 5 µM [mitoTEMPO]</td>
<td>-44.1 ± 0.2</td>
<td>5.9 ± 0.2</td>
</tr>
<tr>
<td>+ 100 µM [NADH] + 1 µM [rotenone]</td>
<td>-45.8 ± 0.4</td>
<td>5.2 ± 0.3</td>
</tr>
<tr>
<td>+ 20 µM [antimycin A]</td>
<td>-46.1 ± 0.4</td>
<td>5.1 ± 0.3</td>
</tr>
<tr>
<td>+ 100 µM [NADH] + 1 mM [malonate]</td>
<td>-45.9 ± 0.4</td>
<td>5.9 ± 0.3</td>
</tr>
<tr>
<td>+ 100 µM [NADH] + 10 mM [azide]</td>
<td>-46.6 ± 0.6</td>
<td>5.5 ± 0.5</td>
</tr>
<tr>
<td>+ 100 µM [NADH] + 300 µM [5-HD]</td>
<td>-39.9 ± 0.2*</td>
<td>6.7 ± 0.1</td>
</tr>
<tr>
<td>+ 200 µM [diazoxide]</td>
<td>-45.1 ± 0.5</td>
<td>7.1 ± 0.5</td>
</tr>
<tr>
<td>+ 100 µM [NADH] + 500 µM [DIDS]</td>
<td>-45.7 ± 0.3</td>
<td>5.1 ± 0.2</td>
</tr>
<tr>
<td>+ 100 µM [NADH] + 0.5 µM [CsA]</td>
<td>-41.2 ± 1.0*</td>
<td>5.3 ± 0.8</td>
</tr>
<tr>
<td>+ 100 µM [NADH] + 50 µM [PK11195]</td>
<td>-41.3 ± 0.7*</td>
<td>5.9 ± 0.5</td>
</tr>
<tr>
<td>+ 100 µM [NADH] + 40 µM [4'-CD]</td>
<td>-45.6 ± 0.5</td>
<td>6.0 ± 0.4</td>
</tr>
<tr>
<td>+ 500 µM [FGIN-1-27]</td>
<td>-46.7 ± 0.4</td>
<td>5.4 ± 0.3</td>
</tr>
<tr>
<td>+ 100 µM [NADH] + 500 µM [FGIN-1-27]</td>
<td>-46.7 ± 0.4</td>
<td>5.4 ± 0.3</td>
</tr>
</tbody>
</table>

Data were shown as mean ± SEM, n is sample number. For $V_{1/2}$, # P<0.05 vs. SCN5A, and *P<0.05 vs. + 100 µM [NADH].
References


