Molecular Medicine

Cardiac Na\(^+\) Current Regulation by Pyridine Nucleotides


Rationale: Mutations in glycerol-3-phosphate dehydrogenase 1-like (GPD1-L) protein reduce cardiac Na\(^+\) current (\(I_{\text{Na}}\)) and cause Brugada Syndrome (BrS). GPD1-L has >80% amino acid homology with glycerol-3-phosphate dehydrogenase, which is involved in NAD-dependent energy metabolism.

Objective: Therefore, we investigated whether the cardiac Na\(^+\) channel might be modulated by alterations in metabolism. Therefore, we investigated whether the cardiac Na\(^+\) channel might be modulated by alterations in metabolism.

Methods and Results: HEK293 cells stably expressing Na\(_{\text{a,1.5}}\) and rat neonatal cardiomyocytes were used. The influence of NADH/NAD\(^+\) on arrhythmic risk was evaluated in wild-type or SCN5A\(^{+/−}\) mouse heart. A280V GPD1-L caused a 2.48±0.17-fold increase in intracellular NADH level (\(P<0.001\)). NADH application or cotransfection with A280V GPD1-L resulted in decreased \(I_{\text{Na}}\) (0.48±0.09 or 0.19±0.04 of control group, respectively; \(P<0.01\)), which was reversed by NAD\(^+\), chelerythrine, or superoxide dismutase. NAD\(^+\) antagonism of the Na\(^+\) channel downregulation by A280V GPD1-L or NADH was prevented by a protein kinase (PK)A inhibitor, PKAI6–22. The effects of NADH and NAD\(^+\) were mimicked by a phorbol ester and forskolin, respectively. Increasing intracellular NADH was associated with an increased risk of ventricular tachycardia in wild-type mouse hearts. Extracellular application of NAD\(^+\) to SCN5A\(^{+/−}\) mouse hearts ameliorated the risk of ventricular tachycardia.

Conclusions: Our results show that Nav1.5 is regulated by pyridine nucleotides, suggesting a link between metabolism and \(I_{\text{Na}}\). This effect required protein kinase C activation and was mediated by oxidative stress. NAD\(^+\) could prevent this effect by activating PKA. Mutations of GPD1-L may downregulate Nav1.5 by altering the oxidized to reduced NAD(H) balance. (Circ Res. 2009;105:737-745.)

Key Words: arrhythmias ■ electrophysiology ■ ion channels ■ sudden death

Brugada syndrome (BrS) is characterized by an electrocardiographic pattern of ST-segment elevation in the right precordial leads and an increased risk of sudden cardiac death.\(^1\) BrS has been associated with cardiac sodium channels (SCN5A or Na\(_{\text{a,1.5}}\)) mutations causing decreased sodium current (\(I_{\text{Na}}\)).\(^2\)–\(^6\) We have reported that the A280V mutation in the glycerol-3-phosphate dehydrogenase 1-like (GPD1-L) causes BrS by reducing \(I_{\text{Na}}\).\(^7\) Other mutations in GPD1-L have also been linked to reduced \(I_{\text{Na}}\) and sudden infant death syndrome.\(^8\)

GPD1-L is highly expressed in heart tissue and has a significant degree of homology (81% at the amino acid level) with glycerol-3-phosphate dehydrogenase (GPD).\(^7\) The GPD family of genes is involved in NAD-dependent energy metabolism. Therefore, we investigated whether the cardiac Na\(_{\text{a,1.5}}\) channel might be modulated by alterations in NAD(H) and whether these changes might explain the effects of GPD1-L mutations on \(I_{\text{Na}}\).

Although regulation by pyridine nucleotides would be a novel finding, Na\(_{\text{a,1.5}}\) is known to be upregulated by protein kinase (PK)A and downregulated by PKC activation.\(^9\)–\(^11\) This regulation most likely involves channel phosphorylation. In the case of PKA regulation, there is evidence of changes in channel trafficking.\(^12\),\(^13\) Pyridine nucleotides have a number of reported effects that could regulate activity of these kinases. For example, 1 mmol/L NADH enhances superoxide production in smooth muscle by \(\approx2\)-fold,\(^14\) and superoxide production can both be caused by and result in PKA activation.\(^15\) Extracellular NAD\(^+\) results in PKA activation in human granulocytes and osteoblastic cells.\(^16\),\(^17\) Therefore, it is conceivable that pyridine nucleotides would regulate Na\(_{\text{a,1.5}}\) through one or more of these established kinase pathways.

Methods

Cell Culture

Full descriptions of the methods are available in the supplemental material. As previously described,\(^18\) we maintained a human embryonic kidney (HEK) cell line stably expressing the human cardiac Na\(_{\text{a,1.5}}\) channel (SCN5A cells). Expression of Na\(_{\text{a,1.5}}\) was linked to
green fluorescent protein (GFP) expression by an internal ribosomal entry site (SCN5A-IRE5-GFP). Cardiomyocytes were isolated from neonatal rat hearts by collagenase treatment (Worthington Biochemical Corporation, Lakewood, NJ). For whole-cell patch-clamping experiments to study GPDI-L effects on Na\(^{+}\), SCN5A cells or cardiomyocytes were transient transfected with wild-type (WT) or A280V GPDI-L and an expression vector containing red fluorescent protein as described previously. In these experiments, cells expressing both GFP and red fluorescent protein were studied.

**Intracellular NADH and NAD\(^{+}\) Levels**

Intracellular NADH and NAD\(^{+}\) levels ([NAD\(^{+}\)]\(_{i}\) and [NAD\(^{-}\)]\(_{i}\)) were detected by using the EnzyChrom NAD\(^{+}\)/NADH Assay Kit (BioAssay Systems, Hayward, Calif) in SCN5A cells with or without infection of an adenov-associated virus containing WT or A280V GPDI-L as described previously. NAD\(^{+}\) (500 µmol/L) was applied extracellularly ([NAD\(^{-}\)]\(_{i}\)) for 18 hours to WT or A280V GPDI-L groups to detect whether it affected the intracellular NADH level. The intensity difference of the reduced product color, measured at 565 nm at time 0 and 15 minutes later is proportionate to the NADH levels in SCN5A cells after infection of WT or A280V GPDI-L. Figure 1 shows that [NAD\(^{-}\)]\(_{i}\) significantly when compared to WT. A280V cardiomyocytes had similar amounts of NADH after infection of WT or A280V GPDI-L. Thus, NAD\(^{-}\) levels appear to be unaltered in these experiments.

**Effects of Treatments on I\(_{Na}\)**

Na\(^{+}\) currents were measured using the whole-cell patch-clamp technique in voltage-clamp mode at room temperature. Pipettes (1 to 2 MΩ) were filled with a pipette solution containing (in mmol/L): CsCl 80, cesium aspartate 80, EGTA 11, MgCl\(_{2}\) 1, CaCl\(_{2}\) 1, HEPES 10, and Na\(_{2}\)ATP 5 (adjusted to pH 7.4 with CsOH). The bath solution consisted of (in mmol/L): NaCl 130, CsCl 5, CaCl\(_{2}\) 1, 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 from a holding potential of -100 mV was applied to establish the presence of voltage-gated Na\(^{+}\),15 channels. Peak currents obtained during the monophasic action potential (MAP) measurements in control condition. Hearts were then perfused with PES at 180 mmol/L NaCl, 10 mmol/L Na\(^{-}\), and 1 mmol/L Na\(^{+}\)-pyruvate, pH 7.4. Monophasic action potentials (MAPs) were recorded on the left ventricular epicardial surface. A custom-made, paired-platinum electrode was placed on the right ventricle to deliver 2 ms (0.5 to 2 mV, twice the threshold) stimuli (GRASS S48 stimulator) to pace the heart at different intervals. The standard pacing protocol consisted of 8 Hz stimuli (basic cycle length, 125 ms) and was delivered for ~20 minutes to measure MAPs. This was followed by programmed electrical stimulation (PES) consisting of trains of 8 paced beats at 125 ms cycle length (S\(_{1}\)) followed by an extra stimulus (S\(_{2}\)). For detection of SCN5A, the primary antibody (rabbit anti-SCN5A, Alomone Labs, Jerusalem, Israel) was diluted 1:100. Alkaline phosphatase–conjugated goat anti-rabbit IgG secondary antibody (Jackson ImmunoResearch, West Grove, Pa) was diluted 1:7500.

**The Effects of NAD(H) Alterations on Ventricular Arrhythmia**

Hearts from WT mice were isolated and Langendorff-perfused (3 mL/min at 37°C) with control Krebs–Henseleit buffer (in mmol/L: NaCl, 119; NaHCO\(_{3}\), 25; KH\(_{2}\)PO\(_{4}\), 1; MgCl\(_{2}\), 1.2; CaCl\(_{2}\), 1.8; glucose, 10; and sodium pyruvate, pH 7.4). Lactate:pyruvate (10:1) Krebs–Henseleit buffer was similarly composed but of 110 mmol/L NaCl, 10 mmol/L Na\(^{-}\), and 1 mmol/L Na\(^{+}\)-pyruvate, pH 7.4. Monophasic action potentials (MAPs) were recorded on the left ventricular epicardial surface. A custom-made, paired-platinum electrode was placed on the right ventricle to deliver 2 ms (0.5 to 2 mV, twice the threshold) stimuli (GRASS S48 stimulator) to pace the heart at different intervals. The standard pacing protocol consisted of 8 Hz stimuli (basic cycle length, 125 ms) and was delivered for ~20 minutes to measure MAPs. This was followed by programmed electrical stimulation (PES) consisting of trains of 8 paced beats at 125 ms cycle length (S\(_{1}\)) followed by an extra stimulus (S\(_{2}\)). Intervals between S\(_{1}\) and S\(_{2}\) were gradually decreased by 1 ms each sweep until S\(_{2}\) overlaid on the 8th S\(_{1}\). PES was repeated 3 times each before and after drug application in each heart. Average APD\(_{90}\) (action potential duration at 90% repolarization) was calculated by averaging 240 MAPs recorded at 3 different locations on each heart (80 MAPs at each location).

Hearts from mice genetically modified to ablate one allele of SCN5A were isolated and Langendorff-perfused (3 mL/min at 37°C). This was followed by assessing arrhythmia using PES and MAP measurements in control condition. Hearts were then perfused with NAD(H), containing Krebs–Henseleit buffer for 20 minutes, and measurements were repeated.

**Statistical Analysis**

Data are shown as means±SEM. Determinations of statistical significance were performed with ANOVAs with Bonferroni testing for comparisons of multiple means. A value of P<0.05 was considered statistically significant.

**Results**

A280V GPDI-L Increased [NAD\(^{-}\)]\(_{i}\)

We measured [NAD\(^{-}\)]\(_{i}\) and [NAD\(^{+}\)]\(_{i}\) in SCN5A cells after infection of WT or A280V GPDI-L. Figure 1 shows that control and WT GPDI-L groups had similar amounts of [NAD\(^{-}\)]\(_{i}\), as compared with untreated cells. On the other hand, [NAD\(^{-}\)]\(_{i}\) was increased 2.48±0.17-fold in A280V GPDI-L group. Both WT and A280V GPDI-L groups reduced [NAD\(^{+}\)]\(_{i}\), significantly when compared to controls.
untreated SCN5A cells (data not shown). Because of subsequent findings that [NAD\(^+\)]\(_i\) could prevent the changes in \(I_{Na}\) mediated by A280V GPD1-L or NADH, we measured the effect of [NAD\(^+\)]\(_i\) on [NADH]. Incubation of 500 \(\mu\)mol/L [NAD\(^+\)]\(_i\) with the WT GPD1-L group did not alter [NADH], compared with control and WT GPD1-L groups. Nevertheless, incubation with [NAD\(^+\)]\(_i\) prevented the increase of [NADH], by A280V GPD1-L.

**Increasing [NADH]\(_i\) Reduced \(I_{Na}\)**

Because A280V GPD1-L altered [NADH] levels, we tested whether increased [NADH] contributed to a reduction in \(I_{Na}\) current. Figure 2A shows examples of \(I_{Na}\) traces obtained with SCN5A cells, which are reduced by 100 \(\mu\)mol/L [NADH]. Figure 2B shows the dose dependence of the NADH effect on \(I_{Na}\). Significantly reduced \(I_{Na}\) was observed with 20 to 1000 \(\mu\)mol/L [NADH] within the biologically relevant range.\(^{24,25}\) The effect reached a maximum at 100 \(\mu\)mol/L [NADH], with a peak current at ~30 mV of 0.54±0.04 of control (\(P<0.01\)). The time course for the NADH effect on \(I_{Na}\) was rapid, suggesting a posttranslational effect. Within 2 to 4 minutes, the NADH effect of decreasing \(I_{Na}\) became stable and durable, lasted for more than 15 minutes. The peak current–voltage relationships of the control SCN5A group and 3 doses of [NADH] are shown in Figure 2C. NADH only slightly affected channel gating parameters in a manner likely too small to explain the reduction in current (Figure 2D; also see the Online Data Supplement, available at http://circres.ahajournals.org). Furthermore, macroscopic inactivation was unaffected by altering [NADH].

With rat neonatal cardiomyocytes, we observed similar effects of [NADH] on reducing \(I_{Na}\) (Figure 2E). With 500 \(\mu\)mol/L [NADH]\(_i\), \(I_{Na}\) was decreased to 0.48±0.08 of the control myocyte group (\(P<0.001\)). Transfection of A280V GPD1-L to myocytes decreased \(I_{Na}\) to 0.19±0.04 of control group (\(P<0.001\)), consistent with our previous observation in SCN5A cells with cotransfection of A280V GPD1-L.\(^7\) Myocytes appeared somewhat less sensitive to pyridine nucleotides than were SCN5A cells, because 20 \(\mu\)mol/L of [NADH], was enough to alter \(I_{Na}\) significantly in the model cell type.

Quantitative real-time PCR was undertaken to evaluate the possibility of alterations in mRNA transcription or stability. We did not observe any reductions in SCN5A mRNA abundances when SCN5A cells were transfected with WT or A280V GPD1-L or treated with extracellular pyridine nucleotides. The mRNA abundances were 98.0±3.3%, 104.2±5.3%, 102.2±2.5%, 96.9±2.1%, and 96.2±2.0% for the control, WT GPD1-L, A280V GPD1-L, NADH, and NAD\(^+\) groups, respectively (\(P>0.05\), consistent with a posttranscriptional regulation of \(I_{Na}\) by A280V GPD1-L or NADH.

**Antagonism of the NADH Effect**

Because NADH is in a redox couple with NAD\(^+\), we tested whether NAD\(^+\) could reverse the NADH effect on \(I_{Na}\). SCN5A cells were incubated with different doses of [NAD\(^+\)]\(_i\) (50 to 1000 \(\mu\)mol/L) for ~18 hours before the patch-clamp recording. With 100 \(\mu\)mol/L [NADH] added in the pipette solution, the expected reduction of \(I_{Na}\) was blocked in a dose-dependent manner by [NAD\(^+\)]\(_i\) (Figure 3A). Internal NAD\(^+\) had a similar effect but at lower doses (data not shown).

Because PKC has been reported to acutely downregulate \(I_{Na}\)\(^10\) and NADH is a substrate for the NADH oxidase,\(^26\) we tested whether PKC and oxidative stress were involved in NADH regulating Na\(_1.5\). Chelerythrine inhibits the PKC catalytic domain\(^27\) and PKC translocation to the membrane.\(^28\) As shown in Figure 3B, both chelerythrine (5 \(\mu\)mol/L) and superoxide dismutase (SOD) (3 \(\mu\)mol/L) blocked the decrease in \(I_{Na}\) seen with [NADH]. Neither agent affected \(I_{Na}\) when applied in the absence of [NADH], (0.77±0.10 and 1.02±0.03 of control group, respectively, \(P>0.05\)). The \(I_{Na}\) reduction seen with [NADH] could be recapitulated by a PKC activator, PMA. As shown in Figure 3C, PMA (30 nmol/L) caused a decrease of \(I_{Na}\) (0.47±0.05 of control group, \(P<0.01\)), which was prevented by 5 \(\mu\)mol/L SOD (0.75±0.01 of control group, \(P>0.05\)).

Activation of PKA increases cardiac Na\(^+\) current acutely.\(^11,12,29\) Therefore, we investigated whether PKA was involved in the signaling pathway by which NAD\(^+\) antagonized the downregulation of \(I_{Na}\) by NADH. As before, [NAD\(^+\)]\(_i\) (500 \(\mu\)mol/L) was applied to SCN5A cells or transfected cells (WT and A280V GPD1-L) for an ~18-hour incubation. PKAI\(_{6,22}\) (100 nmol/L), a specific inhibitor for PKA,\(^30\) prevented the [NAD\(^+\)]\(_i\) antagonism of [NADH] on reducing \(I_{Na}\) (Figure 3D). PKAI\(_{6,22}\) alone at the same dose had no effect on \(I_{Na}\) (1.05±0.15 of the A280V GPD1-L and 0.99±0.32 of the SCN5A groups, respectively, \(P>0.05\)). Channel gating was relatively unaffected between experimental conditions (Online Table II). As expected, when the NAD\(^+\) effect was mediated by PKA, 1 \(\mu\)mol/L forskolin, a PKA activator, blocked the NADH effect on reducing \(I_{Na}\) (Figure 3D), whereas it showed no influence on \(I_{Na}\) when applied alone (0.88±0.05 of the control group, \(P>0.05\)). Application of [NAD\(^+\)]\(_i\), [NAD\(^+\)]\(_i\)+PKAI\(_{6,22}\), chelerythrine, and SOD had the comparable effects on the reduction in \(I_{Na}\) mediated by A280V GPD1-L (Figure 3E). In summary, these experiments suggest that PKC activation and superox-
ide are involved in NADH downregulation of Na\(_{\text{v}1.5}\), mutant GPD1-L most likely has its effect through a similar mechanism, and oxidative stress is a downstream of PKC activation.

**Surface Expression of Na\(_{\text{v}1.5}\) Is Unchanged by Treatments Reducing I\(_{\text{Na}}\)**

Expression of Na\(_{\text{v}1.5}\) near the surface membrane was monitored by confocal microscopy and biotinylation. Both methods showed that Na\(_{\text{v}1.5}\) membrane expression was not affected by incubation with PMA or lactate:pyruvate (Figure 4), conditions known to increase intracellular NADH level\(^{20-22}\) and reduce I\(_{\text{Na}}\) (0.54±0.04 of control, \(P<0.01\)).

**The Effects of NAD(H) Alterations on Ventricular Arrhythmia**

If increased [NADH] reduces I\(_{\text{Na}}\), then elevated [NADH], might be associated with increased arrhythmic risk. Because NADH is impermeable to the sarcolemma, external lactate:pyruvate (10:1) was used to increase [NADH]. The average action potential duration was not significantly changed following 20 minutes of perfusion with lactate:pyruvate versus control (38±2 versus 39±3 ms, respectively). PES failed to induce ventricular tachycardia (VT) in hearts perfused with control Krebs–Henseleit buffer (Figure 5A). When PES was repeated following 20 minutes perfusion with lactate:pyruvate buffer, VT was induced in 2 of 5 hearts (Figure 5B). The arrhythmogenic effect was reversed when control Krebs–Henseleit buffer was reintroduced (Figure 5C).

We tested the biological significance of our findings about NAD\(^+\) amelioration of reduced I\(_{\text{Na}}\) by using a gene-targeted mouse in which one allele of the cardiac Na\(_{\text{v}1.5}\) channel has been ablated (SCN5A\(^{-/-}\)).\(^{23}\) This model shows decreased I\(_{\text{Na}}\) and is characterized by a high proclivity toward inducible VT.
MAPs were recorded from 6 SCN5A+/− mice and 2 WT mouse hearts. Each MAP consists of a rapid upstroke and smooth repolarization phase giving a triangular shape action potential that was similar to MAPs observed previously in murine heart.31,32 The morphology of MAPs was similar to transmembrane action potentials recorded by the patch-clamp technique (Figure 5D and 5E). Perfusion for 20 minutes with 100 μmol/L [NADH]o produced no significant change in
MAP duration or morphology (Figure 5F). Mean values of APD<sub>90</sub> from 6 SCN5A<sup>−/−</sup> hearts after [NAD<sup>+</sup>]o perfusion were close to the control value (29.9 ± 1.7 ms in control versus 27.9 ± 1.9 ms in 100 μmol/L [NAD<sup>+</sup>]o, P = 0.46), as shown in Figure 5G.

To assess the inducibility of ventricular arrhythmia, PES was applied as described in the methods. PES induced multiple episodes of VT of varying durations (0.5 to 48 seconds) in 6 of 6 SCN5A<sup>−/−</sup> hearts (Figure 5H) but not in WT hearts (n=2). This is consistent with previous observation that a decrease in sodium conductance in mouse ventricle by disrupting SCN5A causes VT. After 20 minutes of perfusion with 100 μmol/L [NAD<sup>+</sup>]o, PES failed to induce VT in 5 of 6 hearts, suggesting an antiarrhythmic property of [NAD<sup>+</sup>]o in SCN5A<sup>−/−</sup> hearts (Figure 5I). The effect of [NAD<sup>+</sup>]o was reversible. Multiple episodes of PES-induced VTs were observed in 4 of 6 hearts when [NAD<sup>+</sup>]o was removed.

Discussion
Our data demonstrate that the cardiac sodium channel current can be modulated by pyridine nucleotides. Elevated intracellular NADH resulted in a rapid decrease in <i>I<sub>Na</sub></i> in both HEK cells and cardiomyocytes that was large enough to be clinically significant and of a magnitude seen in BrS. The immediacy of the NADH effect on reducing <i>I<sub>Na</sub></i> observed and the lack of change in mRNA abundances under various experimental conditions suggested that the effect of NADH was posttranscriptional. Membrane expression of SCN5A showed no changes, suggesting that decreased <i>I<sub>Na</sub></i> may be the result of changes in channel gating instead of a decrease of available channels in the membrane. The reduction in current could be prevented by SOD or a PKC inhibitor. PKC-mediated phosphorylation of the channel has been previously reported to result in immediate downregulation of Nav1.5 channels. SOD prevented the downregulation of current in the presence of a PKC activator, suggesting that superoxide was downstream of PKC activation.

NADH is in a redox couple with NAD<sup>+</sup>. Internally or externally applied NAD<sup>+</sup> antagonized the downregulation of current seen with a rise of internal NADH. Nevertheless, the NAD<sup>+</sup> effect did not seem to occur by the same signaling mechanism as did NADH and could be recapitulated by a PKA activator or prevented by a PKA inhibitor. This is consistent with the known effect of NAD<sup>+</sup> to activate PKA in human granulocytes and osteoblastic cells and of PKA activation to increase sodium channel current. A general scheme for pyridine regulation of the sodium channel is presented in Figure 6.

Changes in the Na<sup>+</sup> current induced by pyridine nucleotides are consistent with alterations observed in other channels. Tipparaju and colleagues have reported that NAD(P)H to NAD(P)<sup>+</sup> ratio regulates K<sup>+</sup> currents, although the regulation mostly affects gating rather than peak current. Some transient receptor potential currents are increased by NAD in a manner similar to that seen in our experiments. A nonselective cation channel conductance is also increased by NAD<sup>+</sup>. Zima et al showed that cytosolic NADH inhibited cardiac sarcoplasmic reticulum Ca<sup>2+</sup> release channels, whereas NAD<sup>+</sup> had activating effects on this channel. Analogously to our results with the Na<sup>+</sup> channel, NADH has been reported to decrease Ca<sup>2+</sup>-activated K<sup>+</sup> channel currents, whereas NAD<sup>+</sup> increases the current. Although our buffering conditions were considerably stronger, these effects of NAD(H) on local Ca<sup>2+</sup> homeostasis may help explain some of the changes seen in <i>I<sub>Na</sub></i>, because recent data show Na<sub>1.5</sub> is regulated acutely by Ca<sup>2+</sup>.
The findings provide a possible explanation for the mechanism by which GPD1-L mutations result in BrS7 and sudden infant death syndrome. GPD1-L has a high degree of homology with GPD, a protein that mediates cytoplasmic reduction of dihydroacetone to glycerol using NADH as the electron donor. If GPD1-L were to serve a similar function, the expectation would be that mutations could result in decreased function and an increase in [NADH]. This appears
to be the case, because transfection of cells with mutant A280V GPD1-L resulted in an increased [NADH]i level. Other observations suggesting that this GPD1-L mutant may be working through modulation of pyridine nucleotide levels include: (1) elevations of [NADH]i required Na+ current to a comparable extent as A280V GPD1-L did; (2) WT GPD1-L had no effect on [NADH]i level or I_{Na}; and (3) the effect of both A280V GPD1-L and increased [NADH]i on Na_{1,5} could be reversed by NAD+\textsuperscript{+}, PKC inhibitor, or SOD. On the other hand, although a strong case may be made for alterations of the NAD(H) levels mediating the effect of mutant GPD1-L to cause BrS, this assertion needs to be confirmed with other mutations known to cause the disease.

The physiological relevance of pyridine nucleotide regulation of Na_{1,5} is suggested by the experiments in whole hearts showing manipulations in NAD(H) alter arrhythmic risk. Furthermore, our results suggest that NAD\textsuperscript{+} may be a treatment strategy for GPD1-L–mediated BrS or any arrhythmogenic state associated with reduced I_{Na}, if the acute results are sustained over time. The finding that the balance of oxidized and reduced pyridine nucleotides regulates the Na_{1,5} current suggests that the metabolic state of myocytes may influence Na_{1,5} channel. NADH is known to oscillate with mitochondrial injury, as occurs in ischemic myocardial injury, and mitochondrial injury is associated with increased [NADH], and reactive oxygen species levels.\textsuperscript{46–47} Given the acute nature of effects on Na_{1,5} channels, both of these changes could contribute to reduced I_{Na} and arrhythmic risk known to exist with ischemia. Moreover, heart failure is associated with increased oxidative stress, less [NADH], and increased [NAD(H)\textsubscript{i}].\textsuperscript{48–50} The increased [NADH]i levels may contribute to reduced I_{Na} in this condition.\textsuperscript{19,51,52}

In summary, A280V GPD1-L can induce elevation of [NADH]i, which can downregulate I_{Na} acutely through a PKC activation and increased superoxide. This can be antagonized by PKA activation mediated by NAD\textsuperscript{+}, or by application of a PKC inhibitor or SOD. Our results identify a heretofore unknown regulation of cardiac Na_{1,5} channels that may help explain the link between metabolism and arrhythmic risk\textsuperscript{19,53} and may suggest that NAD\textsuperscript{+} could lessen arrhythmic risk resulting from reduced Na_{1,5} current.

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Disclosures

S.C.D. has a patent pending related to this work, oxidized NAD\textsuperscript{+} treatment for arrhythmia.

References


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Supplemental Material

Cardiac Na⁺ Current Regulation by Pyridine Nucleotides

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Materials and Methods

Cell Culture

All chemicals were purchased from Sigma (St. Louis, MO) except for chelerythrine (Alomone Labs, Jerusalem, Israel) and phorbol 12-myristate 13-acetate (PMA, LC Laboratories, Woburn, MA).

As previously described, we maintained a human embryonic kidney (HEK) cell line stably expressing the human cardiac Na\textsubscript{v}1.5 channel (SCN5A cells). Expression of Na\textsubscript{v}1.5 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\textsubscript{2}/5% CO\textsubscript{2} incubator at 37°C. Cardiomyocytes were isolated from neonatal rat hearts by collagenase treatment (Worthington Biochemical Corporation, Lakewook, NJ) using a protocol approved by the Animal Care Committee of the University of Illinois at Chicago. For whole-cell patch clamping experiments to study GPD1-L effects on Na\textsubscript{v}1.5, SCN5A cells or cardiomyocytes were transient transfected with WT or A280V GPD1-L and an expression vector containing red fluorescent protein (RFP) as described previously. In these experiments, cells expressing both GFP and RFP were studied.

Intracellular NADH and NAD\textsuperscript{+} Levels

Intracellular NADH and NAD\textsuperscript{+} levels ([NADH]\textsubscript{i} and [NAD\textsuperscript{+}]\textsubscript{i}) were detected by using the EnzyChrom\textsuperscript{TM} NAD\textsuperscript{+}/NADH Assay Kit (BioAssay Systems, Hayward, CA) in SCN5A cells with or without infection of an adeno-associated virus containing WT or A280V GPD1-L as described previously. NAD\textsuperscript{+} (500 µmol/L) was applied extracellularly ([NAD\textsuperscript{+}]\textsubscript{o}) for 18 h to WT or A280V GPD1-L groups to detect whether it affected the intracellular NADH level. Briefly, this assay is based on an alcohol dehydrogenase cycling reaction, in which a tetrazolium dye is reduced by
NADH in the presence of phenazine methosulfate. The intensity difference of the reduced product color, measured at 565 nm at time zero and 15 min later is proportionate to the NAD\(^+\)/NADH and is not affected by NADP(H). The absorbance was measured using a PowerWaveX™ spectrophotometer (BIO-TEK Instruments, Winooski, VT).

**Effects of Treatments on \(I_{Na}\)**

\(Na^+\) currents were measured using the whole-cell patch clamp technique in voltage-clamp mode at room temperature as previously described.³ Pipettes (1-2 M\(\Omega\)) 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\(_2\)ATP 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 from a holding potential of -100 mV was applied to establish the presence of voltage-gated Na\(_v\)1.5 channels. Peak currents obtained during steps to -20 or -30 mV were used for comparison in determining the relative reduction in \(I_{Na}\). In all recordings, 80% of the series resistance was compensated, yielding a maximum voltage error of ~1 mV. Data were sampled at 20 kHz and later low pass filtered at 5 kHz for analysis. To minimize time-dependent drift in gating parameters, all protocols were initiated 2-5 min after whole-cell configuration was obtained.

**SCN5A RNA Abundance**

Total RNA (from approximately 2 \(\times\) 10\(^6\) number of cells/well) was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA). Quantitative SYBR real time RT-PCR was carried out as described using primer pair HE27F and HSCN5AE28A/R.⁴ \(\beta\)-actin was used as a reference in all cases. The experiment was carried out in triplicate. Briefly, the SCN5A cells were as a control. WT and A280V GPD1-L were transfected into this cell line. [NAD\(^+\)]\(_o\) (300 \(\mu\)mol/L) was added to the
media for 24 h.

**Na⁺ Channel Localization**

Na⁺ channel localization was performed by two methods, fluorescent microscopy and by biotin labeling of sarcolemmal Na,1.5. For imaging, HEK293 cells were transfected with SCN5A-GFP cDNA, in which the cDNA sequence encoding GFP was fused to the C-terminus of SCN5A.1 Cells were incubated with lactate:pyruvate (10 mmol/L:1 mmol/L) to raise intracellular NADH or treated with PMA (30 nmol/L) for 10 min and then fixed for fluorescent imaging with a Zeiss LSM510 META confocal microscope (Carl Zeiss, Jena, Germany). To determine the proportion of SCN5A expression at the sarcolemma, total cell fluorescence and that of the membrane region were compared. For biotin labeling, SCN5A cells were incubated as above for 2-10 min. Biotinylation of cell surface proteins was performed with the Pinpoint Cell Surface Protein Isolation Kit (Pierce, Rockford, IL) as previously described.3 For detection of SCN5A, the primary antibody (rabbit anti-SCN5A, Alomone Labs, Jerusalem, Israel) was diluted 1:100. Alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) was diluted 1:7500. Radiographs were scanned and digitized for analysis using Quantity One quantification software (BioRad Laboratories).

**The Effects of NAD(H) Alterations on Ventricular Arrhythmia**

Hearts from wild-type mice were isolated and Langendorff-perfused (3 mL/min at 37° C) with control Krebs-Henseleit buffer (in mmol/L: 119 NaCl, 25 NaHCO₃, 4 KCl, 1.2 KH₂PO₄, 1 MgCl₂, 1.8 CaCl₂, 10 glucose and 2 sodium pyruvate, pH 7.4). Lactate:pyruvate (10:1) Krebs-Henseleit buffer was similarly composed but of 110 mmol/L NaCl, 10 mmol/L Na⁺-lactate and 1 mmol/L Na⁺-pyruvate; pH 7.4. Hearts were perfused with control Krebs-Henseleit buffer for 10 min before recording electrophysiological parameters. Then, hearts were perfused with lactate:pyruvate
buffer for 20 min and electrophysiological parameters were recorded again. Finally, control Krebs-Henseleit buffer was re-perfused and electrophysiological parameters were reassessed. Monophasic action potentials (MAPs) were recorded on the left ventricular epicardial surface. MAP signals were amplified, band-pass filtered (0.5 Hz to 1 kHz: Neurolog NL900D), digitized (1401, Cambridge Electronic Design, Cambridge, UK) and acquired using Spike 2.5 (Cambridge Electronic Design) at a 5-kHz sampling rate. A custom made, paired-platinum electrode was placed on the right ventricle to deliver 2 ms (0.5-2 mV, twice the threshold) stimuli (GRASS S48 stimulator) to pace the heart at different intervals. The standard pacing protocol consisted of 8 Hz stimuli (basic cycle length, BCL 125 ms) and was delivered for ~20 min to measure MAPs. This was followed by programmed electrical stimulation (PES) consisting of trains of eight paced beats at 125 ms BCL (S₁) followed by an extra stimulus (S₂). Intervals between S₁ and S₂ were gradually decreased by 1 ms each sweep until S₂ overlaid on the 8th S₁. PES was repeated three times each before and after drug application in each heart. Average APD₉₀ (action potential duration) was calculated by averaging 240 MAPs recorded at three different locations on each heart (80 MAPs at each location).

Hearts from mice genetically modified to ablate one allele of SCN5A⁵ were isolated and Langendorff-perfused (3 ml/min at 37°C) with Krebs-Henseleit solution and allowed to beat intrinsically for 10 min. This was followed by assessing arrhythmia using PES and MAP measurements in control condition. Hearts were then perfused with [NAD⁺]₀ containing Krebs-Henseleit buffer for 20 min, and measurements were repeated.

**Statistical Analysis**

Data are shown as the mean ± SEM. Determinations of statistical significance were performed with ANOVAs with Bonferroni testing for comparisons of multiple means. A value of P<0.05 was considered statistically significant.
Online Table I. Parameters of voltage dependence of steady state activation/inactivation of SCN5A group and NADH groups at different doses.

<table>
<thead>
<tr>
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<th>SSA</th>
<th></th>
<th>SSI</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{1/2}$, mV</td>
<td>$k$, mV</td>
<td>$n$</td>
<td>$V_{1/2}$, mV</td>
</tr>
<tr>
<td>SCN5A</td>
<td>-44.7 ± 0.2</td>
<td>5.7 ± 0.1</td>
<td>14</td>
<td>-72.9 ± 0.3</td>
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<tr>
<td>+ 10 µmol/L NADH</td>
<td>-44.6 ± 0.5</td>
<td>5.0 ± 0.4</td>
<td>9</td>
<td>-76.0 ± 0.3*</td>
</tr>
<tr>
<td>+ 20 µmol/L NADH</td>
<td>-44.5 ± 0.4</td>
<td>5.5 ± 0.3</td>
<td>10</td>
<td>-78.5 ± 0.4*</td>
</tr>
<tr>
<td>+ 50 µmol/L NADH</td>
<td>-45.7 ± 0.6</td>
<td>5.3 ± 0.4</td>
<td>13</td>
<td>-79.6 ± 0.4*</td>
</tr>
<tr>
<td>+ 100 µmol/L NADH</td>
<td>-44.5 ± 0.3</td>
<td>5.5 ± 0.2</td>
<td>16</td>
<td>-75.6 ± 0.3*</td>
</tr>
<tr>
<td>+ 500 µmol/L NADH</td>
<td>-45.6 ± 0.5</td>
<td>5.7 ± 0.4</td>
<td>14</td>
<td>-74.9 ± 0.9</td>
</tr>
<tr>
<td>+ 1000 µmol/L NADH</td>
<td>-46.7 ± 0.7</td>
<td>5.3 ± 0.2</td>
<td>12</td>
<td>-76.2 ± 0.4*</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SEM. *P<0.01 versus SCN5A.
Online Table II. Parameters of voltage dependence of steady state activation/inactivation of GPD1-L (WT, A280V and application of NAD\(^+\), and PKAI_{6-22}).

<table>
<thead>
<tr>
<th></th>
<th>SSA</th>
<th></th>
<th>SSI</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{1/2}$, mV</td>
<td>$k$, mV</td>
<td>$n$</td>
<td>$V_{1/2}$, mV</td>
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<td>WT GPD1-L</td>
<td>-38.3 ± 0.5</td>
<td>6.0 ± 0.5</td>
<td>8</td>
<td>-78.3 ± 0.6</td>
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<tr>
<td>A280V GPD1-L</td>
<td>-34.8 ± 0.5*</td>
<td>6.9 ± 0.4</td>
<td>16</td>
<td>-77.4 ± 0.6</td>
</tr>
<tr>
<td>A280V + 500 µmol/L [NAD(^+)]</td>
<td>-31.9 ± 0.4†</td>
<td>6.2 ± 0.4</td>
<td>13</td>
<td>-78.9 ± 0.5</td>
</tr>
<tr>
<td>A280V + 500 µmol/L [NAD(^+)]  + 100 nmol/L [PKAI_{6-22}]</td>
<td>-40.4 ± 0.5‡</td>
<td>7.9 ± 0.4</td>
<td>10</td>
<td>-82.3 ± 0.4‡</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SEM. *P<0.01 versus WT GPD1-L; †P<0.01 versus A280V GPD1-L; ‡P<0.05 versus A280V + 500 µmol/L [NAD\(^+)\].
References


