Regulation of the Na+/Ca2+ Exchanger by Pyridine Nucleotide Redox Potential in Ventricular Myocytes

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Background: Regulation of the sarcolemmal Na+/Ca2+ exchanger (NCX) modulates cardiac excitation-contraction coupling, and NCX contributes to ischemia-reperfusion injury.

Results: Increased cytosolic NADH inhibits NCX through a ROS-dependent mechanism.

Conclusion: Regulation by cytosolic NADH reveals a novel way that NCX responds to changes in energy metabolism.

Significance: NADH-dependent regulation of NCX regulation represents a potential therapeutic target for ameliorating ischemia-reperfusion injury or chronic cardiovascular disease.

The cardiac Na+/Ca2+ exchanger (NCX) is the major Ca2+ efflux pathway on the sarcolemma, counterbalancing Ca2+ influx via L-type Ca2+ current during excitation-contraction coupling. Altered NCX activity modulates the sarcoplasmic reticulum Ca2+ load and can contribute to abnormal Ca2+ handling and arrhythmias. NADH/NAD+ is the main redox couple controlling mitochondrial energy production, glycolysis, and other redox reactions. Here, we tested whether cytosolic NADH/NAD+ redox potential regulates NCX activity in adult cardiomyocytes. NCX current (I_{NCX}) measured with whole cell patch clamp, was inhibited in response to cytosolic NADH loaded directly via pipette or increased by extracellular lactate perfusion, whereas an increase of mitochondrial NADH had no effect. Reactive oxygen species (ROS) accumulation was enhanced by increasing cytosolic NADH, and NADH-induced I_{NCX} inhibition was abolished by the H2O2 scavenger catalase. NADH-induced ROS accumulation was independent of mitochondrial respiration (rotenone-insensitive) but was inhibited by the flavoenzyme blocker diphenylene iodonium. NADPH oxidation was ruled out as the effector because I_{NCX} was insensitive to cytosolic NADPH, and NADH-induced ROS and I_{NCX} inhibition were not abrogated by the specific NADPH oxidase inhibitor gp91ds-tat. This study reveals a novel mechanism of NCX regulation by cytosolic NADH/NAD+ redox potential through a ROS-generating NADH-driven flavoprotein oxidase. The mechanism is likely to play a key role in Ca2+ homeostasis and the response to alterations in the cytosolic pyridine nucleotide redox state during ischemia-reperfusion or other cardiovascular diseases.

The sarcolemmal Na+/Ca2+ exchanger (NCX) mediates the electrogenic exchange of three Na+ for one Ca2+. It operates in either forward mode (Ca2+ efflux) or reverse mode (Ca2+ influx), depending on the membrane potential and ion gradients across the plasma membrane. In the heart, Ca2+ entry via the L-type Ca2+ channel during the action potential triggers a larger amount of Ca2+ release from the sarcoplasmic reticulum (SR) via the ryanodine receptor, mediating cardiac muscle contraction. During relaxation, cytosolic Ca2+ is removed by both the SR Ca2+ ATPase and by forward-mode NCX activity. At steady state, the amount of Ca2+ removal via NCX equals Ca2+ entry via the L-type Ca2+ channel. The contribution of NCX to total Ca2+ removal in the cardiac myocyte is species-dependent, varying from less than 10% in mice and rats to up to 30% in guinea pigs, rabbits, and humans (1). Thus, NCX plays an important role in cardiac physiology by maintaining Ca2+ homeostasis and modulating the action potential morphology because of its electrogenic properties.

Abnormal NCX activity contributes to contractile and electrical dysfunctions of the myocardium, and NCX plays a direct role in Ca2+ overload during ischemia/reperfusion. The rise of intracellular Na+ during ischemia promotes the reverse mode of NCX activity, resulting in Ca2+ influx, and the consequent Ca2+ overload is a key factor for necrotic cell death and ischemic arrhythmia. Decreasing NCX activity by NCX inhibitors (2–4), shRNA (5), or NCX knock out (6, 7) protects the myocardium from ischemia-reperfusion injury. In heart failure, NCX activity is modified by multiple factors, and the consequences of NCX modification on cardiac function are complicated. NCX is up-regulated at mRNA, protein, and functional levels in various heart failure models (8–11). Although increased NCX can have a positive inotropic effect because of more Ca2+ influx during the action potential plateau (12) and compensates for impaired SR Ca2+ ATPase (13), inhibition of NCX activity can have beneficial effects on heart failure by enhancing the SR Ca2+ load (10, 14, 15).

NCX consists of nine transmembrane segments and a large intracellular loop that is essential for regulation of its activity by a large variety of factors, such as Ca2+, phosphatidylinositol 4,5-bisphosphate, and reactive oxygen species (ROS) (16–18). Several lines of evidence suggest a correlation between cardiac NCX activity and energy metabolism (19–22) in which NADH...
tions. NADH/NAD\textsuperscript{+} redox potential determines the activity of enzymes that utilize NADH to donate electrons in both the cytosol and the mitochondrial matrix. In the cytosol, NADH is mainly produced by glycolysis and oxidized either anaerobically by the lactate dehydrogenase reaction or transported indirectly into the mitochondria via a shuttle system to be oxidized by NADH-ubiquinone oxidoreductase (complex I) of the respiratory chain. Although, under physiological conditions, cytosolic NAD\textsuperscript{+}:NADH has been reported to range from 500–1000 (23, 24), under hypoxia or impaired mitochondrial oxidative phosphorylation, the lactate:pyruvate ratio increases, resulting in reduction of the mitochondrial pool. Moreover, chronic pathological conditions such as angina, heart failure, or diabetes involve alterations in glycolysis, oxidative phosphorylation, or blood lactate concentrations (lactic acidosis), which will lead to an altered cytosolic NADH/NAD\textsuperscript{+} ratio.

In this study, we investigate whether an altered cytosolic NADH/NAD\textsuperscript{+} redox potential modulates NCX activity in adult guinea pig ventricular myocytes. We demonstrate that cardiac NCX is inhibited by increased cytosolic NADH and that this effect is mediated by ROS. We further characterize the source of NADH-induced ROS, which is independent of mitochondrial respiration and is prevented by a flavoprotein inhibitor, diphenylene iodonium (DPI). Our findings reveal a novel mechanism of cardiac NCX regulation by cytosolic NADH/NAD\textsuperscript{+} redox potential.

**EXPERIMENTAL PROCEDURES**

**Isolation of Adult Guinea Pig Ventricular Myocytes**—All animal handling and care was performed in accordance with the Guide for Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996) and approved by The Johns Hopkins Animal Care and Use Committee. Ventricular myocytes were enzymatically isolated from adult guinea pig heart as described previously (25) and maintained in DMEM supplemented with 5% FBS, 1% penicillin-streptomycin, and 10 mM HEPES (pH 7.4) in a CO\textsubscript{2} incubator at 37 °C (Fig. 4B).

**Whole-cell Patch Clamp and NCX Current (I\textsubscript{NCX}) Recording**—Myocytes were loaded in a heated chamber (37 °C) on the stage of a fluorescence microscope (Nikon Eclipse TE300) and superfused with Cs-Tyrode’s solution containing 130 mM NaCl, 5 mM CsCl, 1 mM MgCl\textsubscript{2}, 10 mM sodium-HEPES, 2 mM CaCl\textsubscript{2}, and 10 mM glucose (pH 7.4). To block L-type Ca\textsuperscript{2+} channels and Na\textsuperscript{+} -K\textsuperscript{+}-ATPase, 10 mM nitrindipine and strophanthidin, respectively, were applied in Cs-Tyrode’s solution. Myocytes were whole-cell patch-clamped with 2–4 M\textOmega pipettes and equilibrated with a pipette solution containing 120 mM CsCl, 0.5 mM MgCl\textsubscript{2}, 20 mM sodium-HEPES, 5 mM magnesium-ATP, 5 mM 1,2-bis(o-aminophenoxy)ethane-N,N,N',N"-tetraacetic acid (BAPTA), and 3 mM CaCl\textsubscript{2} (pH 7.25). The presence of 5 mM BAPTA and 3 mM CaCl\textsubscript{2} in pipette solution buffered the intracellular Ca\textsuperscript{2+} to 200 nm. After achievement of whole-cell conformation, cells were voltage-clamped at a holding potential of −40 mV. I\textsubscript{NCX} was recorded with families of pulses applied from +80 to −80 mV at 20-mV steps (Fig. 1, inset) elicited nickel-sensitive and nickel-insensitive currents. I\textsubscript{NCX} was defined as the nickel-sensitive component (Fig. 1C) determined by subtracting nickel-insensitive (B) from the total current (A). In Fig. 1D, the currents in response to repeated families of pulses were recorded with control pipette solution and plotted against experimental time (I-t plot). The representative I-t plot of control currents (Fig. 1D) illustrated that, starting from a low base-line level (currents elicited by the first family of pulses), I\textsubscript{NCX} was gradually activated and reached a steady state within 2–3 min, in keeping with previous studies (26).

Application of 5 mM nickel to the perfusate rapidly blocked I\textsubscript{NCX}, leaving only a stable background current.

To test the effects of NADH on I\textsubscript{NCX}, we applied 100 or 400 mM NADH, respectively, in the intracellular pipette solutions and measured I\textsubscript{NCX}. Although 100 mM NADH had no effect on I\textsubscript{NCX} (Fig. 2, A and G), application of 400 mM NADH significantly inhibited I\textsubscript{NCX} (Fig. 2, B, C, and G). Reverse-mode I\textsubscript{NCX} at +80 mV decreased by 66% (4.57 ± 0.26 pA/pF in the control current). As indicated, NADH, NADPH, catalase, or rotenone were added to the intracellular pipette solutions. Stock solutions of NADH, NADPH, and catalase were freshly prepared on the day of the experiment. When testing each compound or condition, NCX currents were compared with those of control and/or NADH groups in the same cell preparation unless specified otherwise.

**Measurement of NADH Auto-fluorescence**—To record NADH in single cells (Figs. 2D and 3A), myocytes were excited at λ\textsubscript{ex} = 360 nm, and NADH auto-fluorescence was collected at λ\textsubscript{em} = 450 nm with a photomultiplier tube (PITI Inc., Birmingham, NJ). The mitochondrial NADH oxidation state was calibrated by applying 4 mM NaCN (CN\textsuperscript{−}) for 100% NADH reduction and 5 mM carbonyl cyanide p-trifluoromethoxyphenylhydrazone for 0% NADH reduction at the end of recording. NADH in myocyte suspensions was measured in a quartz cuvette with a fluorometer (QuantaMaster, PTI, Inc.) with stirring and temperature-controlled at 37 °C (Fig. 4B).

**Measurements H\textsubscript{2}O\textsubscript{2} Production**—To monitor H\textsubscript{2}O\textsubscript{2} accumulation in single cells, myocytes were loaded with 5-(6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate and its fluorescent product of oxidation by H\textsubscript{2}O\textsubscript{2} (DCF), was recorded with excitation at λ\textsubscript{ex} = 485 nm and emission at λ\textsubscript{em} = 530 nm with a photomultiplier tube. The total H\textsubscript{2}O\textsubscript{2} emission in cell suspensions was measured with Amplex UltraRed (Invitrogen). Myocytes were suspended in 2 ml of normal Tyrode’s solution with 50 μM Amplex UltraRed reagent, 5 units/ml horseradish peroxidase, and 10 units superoxide dismutase in a stirred quartz cuvette. Fluorescence intensity was measured with a fluorometer at 37 °C.

**Statistical Analysis**—Values are reported as mean ± S.E. Statistical analysis between I-V plots of I\textsubscript{NCX} and between DCF recordings (Fig. 4A) were performed using multiple linear regression. For average I\textsubscript{NCX} at +80 or −80 mV, unpaired Student’s t test was performed. Reagents were obtained from Sigma-Aldrich unless indicated otherwise.

**RESULTS**

**Inhibition of I\textsubscript{NCX} by NADH**—With a holding potential of −40 mV, a family of pulses from +80 to −80 mV at 20-mV steps (Fig. 1, inset) elicited nickel-sensitive and nickel-insensitive currents. I\textsubscript{NCX} was defined as the nickel-sensitive component (Fig. 1C) determined by subtracting nickel-insensitive (B) from the total current (A). In Fig. 1D, the currents in response to repeated families of pulses were recorded with control pipette solution and plotted against experimental time (I-t plot). The representative I-t plot of control currents (Fig. 1D) illustrated that, starting from a low base-line level (currents elicited by the first family of pulses), I\textsubscript{NCX} was gradually activated and reached a steady state within 2–3 min, in keeping with previous studies (26).

Application of 5 mM nickel to the perfusate rapidly blocked I\textsubscript{NCX}, leaving only a stable background current.
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and 1.57 ± 0.20 pA/pF in 400 μM NADH, p < 0.0001), and forward-mode $I_{\text{NCX}}$ at -80 mV decreased by 70% (-0.79 ± 0.10 pA/pF in the control and -0.24 ± 0.12 pA/pF in 400 μM NADH, p < 0.001) (Fig. 2C). Fig. 2E shows a representative I-t plot with the currents recorded in the presence of 400 μM NADH right after the achievement of whole-cell configuration. In contrast to the gradual activation under control conditions, $I_{\text{NCX}}$ declined with 400 μM NADH in the pipette solution and reached a steady state in about 3 min of recording (Fig. 2E). To test whether NADPH had similar inhibitory effects on $I_{\text{NCX}}$, $I_{\text{NCX}}$ was measured in the presence of 100 or 400 μM NADPH in the pipette solution. NADPH showed no effect on $I_{\text{NCX}}$ at either concentration (Fig. 2, F and G). The cumulative I-V plots of control $I_{\text{NCX}}$ and $I_{\text{NCX}}$ in the presence of NADH or NADPH are shown in Fig. 1G.

To investigate whether increasing endogenous cytosolic NADH has a similar inhibitory effect on $I_{\text{NCX}}$, 1 mM lactate was applied in the perfusate to promote the reduction of NAD$^+$ by lactate dehydrogenase reaction in the cytosol. Following lactate perfusion, NADH autofluorescence in myocytes increased by 20–30% of the total cellular NADH pool (Fig. 3A). $I_{\text{NCX}}$ was markedly inhibited at a time point 5 min after lactate perfusion (Fig. 3B). Compared with control $I_{\text{NCX}}$, the forward mode of $I_{\text{NCX}}$ at -80 mV decreased by 85% (-0.80 ± 0.15 pA/pF in control cells and -0.12 ± 0.07 pA/pF in lactate-treated cells, p < 0.01), and the reverse mode of $I_{\text{NCX}}$ at +80 mV decreased by 74% (5.76 ± 0.72 pA/pF in control cells and 1.52 ± 0.25 pA/pF in lactate-treated cells, p < 0.005).

Next we examined whether increasing mitochondrial NADH had the same effect on $I_{\text{NCX}}$ as that of increasing cytosolic NADH. $I_{\text{NCX}}$ was measured in myocytes in which mitochondrial NADH was fully reduced by applying 4 mM NaCN to the perfusate. The reverse-mode of $I_{\text{NCX}}$ at +80 mV was 4.00 ± 0.07 pA/pF, which was not significantly different from the control shown in Fig. 2G.

Increased ROS by NADH—To determine whether the increased cytosolic NADH/NAD$^+$ redox potential affected intracellular ROS, cumulative H$_2$O$_2$ production was monitored in both single myocytes and cell suspensions. Myocytes loaded with 5-(6)-chloromethyl-2',7'-dichlorohydrofluorescein diace-
FIGURE 2. Effects of NADH and NADPH on \( I_{\text{NCX}} \). A and B, families of representative \( I_{\text{NCX}} \) recorded in the presence of 100 \( \mu \text{M} \) (A) or 400 \( \mu \text{M} \) NADH (B). C, cumulative \( I_{\text{NCX}} \) measured at −80 and +80 mV with (red bars, \( n = 14 \)) and without (black bars, \( n = 22 \)) 400 \( \mu \text{M} \) NADH. *, \( p < 0.001 \); **, \( p < 0.0001 \) compared with the control. D, NADH autofluorescence in a myocyte recorded right after achieving whole-cell configuration showing an increase of NADH during equilibration with a pipette solution containing 400 \( \mu \text{M} \) NADH. As indicated, 4 mM CN\(^-\) and 5 \( \mu \text{M} \) carbonyl cyanide \( p\)-trifluoromethoxyphenylhydrazone (FCCP) were applied at the end of the recording to fully reduce and oxidize the NADH pool, respectively. E, representative \( i-t \) plot of currents recorded in the presence of 400 \( \mu \text{M} \) NADH. F, families of representative \( I_{\text{NCX}} \) recorded in the presence of 100 \( \mu \text{M} \) (left panel) and 400 \( \mu \text{M} \) (right panel) NADPH. G, summary I-V plots of average \( I_{\text{NCX}} \) recorded with control solution (\( n = 22 \)), 100 \( \mu \text{M} \) (\( n = 7 \)) or 400 \( \mu \text{M} \) (\( n = 12 \)) NADH and 100 \( \mu \text{M} \) (\( n = 7 \)) or 400 \( \mu \text{M} \) (\( n = 6 \)) NADPH. #, \( p < 0.01 \) compared with control \( I_{\text{NCX}} \).

FIGURE 3. Effects of lactate on NADH and \( I_{\text{NCX}} \). A, NADH autofluorescence showing an increase of NADH during lactate perfusion. CN\(^-\) and carbonyl cyanide \( p\)-trifluoromethoxyphenylhydrazone (FCCP) are applied as indicated. B, summary I-V plots of average \( I_{\text{NCX}} \) recorded with (\( n = 7 \)) or without (\( n = 5 \)) lactate treatment. *, \( p < 0.001 \).
Inhibitory Effect of NADH Is Independent of Mitochondrial Respiration—The mitochondrial electron transport chain is a major source of ROS production in cells. To investigate whether the electron transport chain plays a role in NADH-induced ROS, H$_2$O$_2$ production induced by lactate was monitored in the presence of rotenone in myocyte suspensions. Rotenone did not block the increased H$_2$O$_2$ production following lactate application (Fig. 6A), suggesting that NADH-induced ROS is independent of the electron transport chain. $I_{\text{NCX}}$ was further measured in the presence of NADH with or without rotenone. Rotenone did not prevent NADH-induced $I_{\text{NCX}}$ inhibition. $I_{\text{NCX}}$ was not significantly different between groups with and without rotenone (at -80 mV, $-0.31 \pm 0.16$ pA/pF with rotenone, $p = 0.05$ in the presence of DPI ($n = 5$) and DPI, a nonspecific NOX inhibitor. In myocytes treated with 3 mM gp91ds-tat (AnaSpec, Inc., Fremont, CA), to examine the role of NOX2. The NADH inhibitory effect on $I_{\text{NCX}}$ was still evident in myocytes dialyzed with 3 mM gp91ds-tat (Fig. 7A), and the response was not significantly different from that of NADH alone. We then tested the effect of DPI, a nonspecific NOX inhibitor. In myocytes treated with 3 mM DPI, $I_{\text{NCX}}$ inhibition by NADH was abrogated (Fig. 7A). Moreover, DPI also inhibited the lactate-induced ROS increase in myocyte suspensions (the ratio of Amplex oxidation rate after lactate administration to that before lactate administration was 1.11 $\pm$ 0.05 in the presence of DPI ($n = 6$) versus 2.31 $\pm$ 0.44 without DPI ($n = 6$) (Fig. 7, B and C). These results suggest that a cytoplasmic NADH-dependent flavoenzyme other than NOX2 or complex I is implicated in the modulation of $I_{\text{NCX}}$.

DISCUSSION

In this study, we investigated the role of NADH/NAD$^+$ redox potential in the modulation of sarcolemmal NCX in isolated guinea pig ventricular myocytes. We found that increasing cytosolic NADH via patch clamp (400 $\mu$M) or by lactate treatment significantly inhibited $I_{\text{NCX}}$. This inhibition of NCX was mediated by H$_2$O$_2$. We also found that ROS induced by NADH
was independent of mitochondrial respiration. These findings suggest that, during metabolic stress in cardiomyocytes, increased cytosolic NADH can play a critical role in both oxidative stress and Ca$^{2+}$/H$^{+}$ homeostasis.

The NADH/NAD$^{+}$ ratio is an important component of cellular redox state and regulates energy metabolism in the cytoplasm by controlling the activity of several enzymatic reactions in the cytosol. Alterations in NADH/NAD$^{+}$ ratio not only reflect abnormal redox and metabolic conditions but can also play a primary role in the regulation of ion channels. Recent studies demonstrated that NADH inhibits the Na$^{+}$ current (27, 28) and increases spontaneous SR Ca$^{2+}$ release (29) in cardiomyocytes. A sustained elevation of cytosolic NADH can occur under ischemic or hypoxic conditions, which was mimicked in this work by raising cytoplasmic NADH via the patch pipette or by increasing the NADH/NAD$^{+}$ redox potential with lactate treatment, leading to a marked inhibition of $I_{\text{NCX}}$. Inhibition of NCX by NADH is, therefore, likely to be an important

**FIGURE 5. Role of ROS in $I_{\text{NCX}}$ inhibition.** A, summary I-V curves for $I_{\text{NCX}}$ measured in the presence of 400 $\mu$M NADH with ($n$ = 6) or without ($n$ = 4) catalase and for DMNQ-treated myocytes ($n$ = 7) with control pipette solution. *, $p < 0.05$ compared with that without catalase. B, $I_{\text{NCX}}$ measured at −80 and +80 mV in the presence of NADH with (red) or without (black) catalase and in DMNQ-treated myocyte with control pipette solution (blue). #, $p < 0.01$ compared with that without catalase.

**FIGURE 6. Lack of effect of rotenone on lactate-induced ROS and NADH-induced $I_{\text{NCX}}$ inhibition.** A, the Amplex Red signal was monitored in the presence of 5 $\mu$M rotenone. 5 mM lactate was added as indicated. B, cumulative $I_{\text{NCX}}$ at −80 and +80 mV measured in the presence of 400 $\mu$M NADH with ($n$ = 6) or without ($n$ = 3) 1 $\mu$M rotenone (Rot). A.U., arbitrary fluorescence units.

**FIGURE 7. Effects of DPI and gp91ds-tat.** A, summary I-V curves of $I_{\text{NCX}}$ measured with control pipette solution ($n$ = 4) or pipette solutions containing 400 $\mu$M NADH in control myocytes ($n$ = 6) or in myocytes preincubated with 3 $\mu$M gp91ds-tat (gp91) ($n$ = 6) or 3 $\mu$M DPI ($n$ = 7). *, $p < 0.01$ compared with the control. B, representative Amplex Red signal monitored in the presence of 5 $\mu$M DPI. 5 mM lactate did not alter the Amplex Red oxidation rate in the presence of DPI. C, summary of Amplex oxidation rate following lactate (lac) administration with ($n$ = 6) and without DPI, which was normalized to the rate before lactate administration. A.U., arbitrary fluorescence units.
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mechanism for \( \text{Ca}^{2+} \) regulation in the heart when energy metabolism is compromised. This effect would occur in parallel with other factors that modulate NCX during changes in metabolism. For example, ATP depletion causes a significant decrease in cardiac NCX activity (19, 20, 30, 31), and lactate accumulation not only increases cytosolic NADH but also mediates acidosis. Decreased pH inhibits NCX activity (32), which is fully reversible after pH normalization (33). In our study, acidosis is not expected to contribute to the observed lactate- or NADH-mediated \( I_{\text{NCX}} \) inhibition because cytosolic pH was buffered by dialysis with the pipette solution containing 10 mM HEPES at pH 7.25. Increased plasma lactate is associated with vigorous exercise, hypoxia, or ischemia. It is also observed in patients with metabolic syndrome, diabetes, congestive heart failure, and various other diseases (34–36). In a study of ischemia/reperfusion in the isolated guinea pig heart, Bassenge et al. (37) showed that lactate stimulated ROS production several-fold, whereas pyruvate inhibited postischemic ROS production up to 80% in a dose-dependent way. The finding that lactate or cytosolic NADH induce an increase in ROS and inhibit \( I_{\text{NCX}} \) may shed light on oxidative stress and abnormal \( \text{Ca}^{2+} \) handling in the heart under these conditions.

The observation of NADH-induced ROS production in our study is consistent with others (27–29, 37–39). However, the mechanism is not well understood. A mechanism of NADH-induced ROS accumulation was proposed in a recent study (27, 28) in HEK cells and neonatal rat ventricular myocytes, demonstrating that NADH inhibited cardiac \( I_{\text{Na}} \) current (\( I_{\text{Na}} \)) by evoking mitochondrial overproduction of ROS. This effect was dependent on PKC activation and mitochondrial function and was blocked by inhibitors of complexes I and II, rotenone and malonate, respectively. However, in the present study in adult guinea-pig myocytes, NADH-induced ROS and NCX inhibition were not reversed by rotenone, suggesting a different mechanism of NADH-induced ROS, likely generated by a cytosolic source. Different metabolic requirements can lead to remarkable differences in mitochondrial function and composition (40, 41) and energy supply in the adult heart predominantly depends on fatty acid oxidation, whereas neonatal rat ventricular myocytes or HEK cells are more dependent on glycolysis. It is therefore possible that adult cardiomyocytes have different mechanisms to handle and respond to cytosolic NADH increases. For example, the adult heart has a much lower NADH shuttle capacity compared with the neonatal heart (42). In support of a cytosolic source of NADH-induced ROS in the heart, Mohazzab et al. (38) demonstrated that lactate induced superoxide production in calf cardiomyocytes by lucigenin assay. The inability of lucigenin to detect antimycin A-induced mitochondrial superoxide confirmed the cytosolic origin of lactate-induced ROS (38), and the NADH-induced ROS in calf myocytes shares several aspects with the findings in our present study: it is rotenone-insensitive but DPI-sensitive, and it utilizes NADH but not NADPH. The sensitivity to DPI suggests that it may be a member of the NAD(P)H oxidase (NOX) family or another flavoprotein-containing NADH oxidoreductase (38). Further studies are necessary to identify the oxidase with specific inhibitors or genetic manipulation of potential candidates.

NCX activity is known to be modified by ROS, but the effects are inconsistent. Reeves et al. (18) demonstrated that NCX activity in bovine sarcolemmal vesicles was stimulated by incubation with FeSO4 and DTT or simultaneous application of GSH (1–2 mM) and GSSG (1–2 mM), suggesting that disulfide bond modification played a role. However, Santacruz-Toloz et al. (43) showed that a mutant NCX without cysteine residues was still stimulated by FeSO4 and DTT. Thus, the modification reaction underlying NCX stimulation remains unresolved. On the other hand, there is evidence showing depressed NCX activity by ROS (17, 44). Dixon et al. (17) demonstrated that ROS-induced NCX inhibition was prevented by superoxide dismutase and catalase. NCX contains 15 endogenous cysteine residues which are potential targets for \( \text{H}_2\text{O}_2 \)-mediated thiol modification. However, measurements of NCX activity after mutations of cysteine residues of the exchanger indicated differential effects of individual cysteines on function (45). Mutations of C20A at the extracellular N terminus and C792A at extracellular loop between transmembrane segments 7 and 8 had a large inhibitory effect, whereas mutations of C383, 387A at the intracellular regulatory loop, and C933A at intracellular C terminus, had large stimulatory effects (45). These data suggest that the mechanisms of NCX modification by ROS could be complicated, depending on ROS levels and the specific site of modification.

In the present study, the inhibitory effect of NADH on NCX was abolished by catalase, indicating that \( \text{H}_2\text{O}_2 \) is the mediator. However, treatment with exogenous \( \text{H}_2\text{O}_2 \) did not significantly stimulate or inhibit NCX activity in our study (data not shown) and those of others (18, 44). The failure of exogenous \( \text{H}_2\text{O}_2 \) to mimic NCX inhibition by NADH implies that NADH-induced \( \text{H}_2\text{O}_2 \) generation by the DPI-sensitive enzyme, and the subsequent NCX inhibition, are highly site-specific and finely regulated processes. \( \text{H}_2\text{O}_2 \) is known to be a signaling molecule that regulates a large variety of protein kinases and phosphatases (46), including PKA and PKC, which have been shown to activate NCX (47, 48), and tyrosine kinases, which have been reported to inhibit NCX current (49). Bulliard et al. (50) reported that, in brain synaptosomes, millimolar concentrations of either the reduced or oxidized forms of NAD(P)H inhibited NCX and observed alterations in protein phosphorylation. Hence, it is possible that modifications other than direct oxidative regulation of the NCX protein could be involved in the regulation of cardiac NCX.

The effects of increased cytosolic NADH on other \( \text{Ca}^{2+} \) handling proteins are not completely understood, but modulation of these proteins by ROS is well established (39, 51). The global impact of NCX inhibition by NADH on \( \text{Ca}^{2+} \) homeostasis will depend on the overall effect of modifications on all of these proteins as well as on the pathological conditions responsible for the increased cytosolic NADH. For example, in acute ischemia, rapid accumulation of NADH occurs in parallel with \( \text{Na}^+ \) overload, which drives the reverse mode NCX, leading to \( \text{Ca}^{2+} \) overload. The inhibition of NCX by NADH, thus, could be a mechanism that would prevent ischemic \( \text{Ca}^{2+} \) overload. In contrast, a chronic increase of cytosolic NADH, and subsequent NCX inhibition, could either preserve SR \( \text{Ca}^{2+} \) load in the context of increased SR-\( \text{Ca}^{2+} \) leak and decreased SR \( \text{Ca}^{2+} \)
ATPase pump activity or promote SR Ca\(^{2+}\) overload by impairing transsarcolemmal Ca\(^{2+}\) efflux.

In summary, we demonstrate that cardiac NCX is inhibited by cystolic NADH in adult myocytes. Increased cystolic NADH promotes a ROS increase through a mechanism that involves a flavoenzyme (i.e., DPI-sensitive) other than NOX2 or complex I of the respiratory chain. NADH was more effective than NADPH in inhibiting \(I_{\text{NCX}}\) and locally produced H\(_2\)O\(_2\) appears to be the key mediator. Because cystolic NADH and/or plasma lactate increases with ischemic and other cardiovascular diseases, this mechanism is likely to contribute to altered Ca\(^{2+}\) homeostasis and oxidative stress under these conditions, therefore representing a potential therapeutic target.

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