Cardiac-Specific Ablation of the Na\(^+\)-Ca\(^{2+}\) Exchanger Confers Protection Against Ischemia/Reperfusion Injury

Kenichi Imahashi, Christian Pott, Joshua I. Goldhaber, Charles Steenbergen, Kenneth D. Philipson, Elizabeth Murphy

Abstract—During ischemia and reperfusion, with an increase in intracellular Na\(^+\) and a depolarized membrane potential, Ca\(^{2+}\) may enter the myocyte in exchange for intracellular Na\(^+\) via reverse-mode Na\(^+\)-Ca\(^{2+}\) exchange (NCX). To test the role of Ca\(^{2+}\) entry via NCX during ischemia and reperfusion, we studied mice with cardiac-specific ablation of NCX (NCX-KO) and demonstrated that reverse-mode Ca\(^{2+}\) influx is absent in the NCX-KO myocytes. Langendorff perfused hearts were subjected to 20 minutes of global ischemia followed by 2 hours of reperfusion, during which time we monitored high-energy phosphates using \(^{31}\)P-NMR and left-ventricular developed pressure. In another group of hearts, we monitored intracellular Na\(^+\) using \(^{23}\)Na-NMR. Consistent with Ca\(^{2+}\) entry via NCX during ischemia, we found that hearts lacking NCX exhibited less of a decline in ATP during ischemia, delayed ischemic contracture, and reduced maximum contracture. Furthermore, on reperfusion following ischemia, NCX-KO hearts had much less necrosis, better recovery of left-ventricular developed pressure, improved phosphocreatine recovery, and reduced Na\(^+\) overload. The improved recovery of function following ischemia in NCX-KO hearts was not attributable to the reduced preischemic contractility in NCX-KO hearts, because when the preischemic workload was matched by treatment with isoproterenol, NCX-KO hearts still exhibited improved postischemic function compared with wild-type hearts. Thus, NCX-KO hearts were significantly protected against ischemia-reperfusion injury, suggesting that Ca\(^{2+}\) entry via reverse-mode NCX is a major cause of ischemia/reperfusion injury. (Circ Res. 2005;97:916-921.)

Key Words: Na\(^+\)-Ca\(^{2+}\) exchange ■ genetically altered mice ■ ischemia/reperfusion injury

A rise in intracellular Na\(^+\) occurs during ischemia followed by a rise in intracellular Ca\(^{2+}\). A substantial proportion of the rise in Ca\(^{2+}\) is dependent on the rise in intracellular Na\(^+\) suggesting an important role for Na\(^+\)-Ca\(^{2+}\) exchange (NCX).\(^1\)\(\sim\)\(^6\) The NCX is a transporter that exchanges 3 Na\(^+\) for 1 Ca\(^{2+}\); it is therefore electrogenic and influenced by membrane potential as well as the Na\(^+\) and Ca\(^{2+}\) gradients. Thus, depending on the Na\(^+\) and Ca\(^{2+}\) gradients and the membrane potential, the exchanger can extrude Ca\(^{2+}\) from the cell (the forward mode) or transport Ca\(^{2+}\) into the cell (reverse mode).\(^7\)\(\sim\)\(^8\) The Na\(^+\)-dependent increase in Ca\(^{2+}\) during ischemia and reperfusion could result from reduced Ca\(^{2+}\) efflux caused by a reduced Na\(^+\) gradient or from Ca\(^{2+}\) entry via reverse-mode NCX.\(^4\) If the rise in Ca\(^{2+}\) is primarily attributable to reduced Ca\(^{2+}\) efflux, then inhibition of NCX might increase the rise in Ca\(^{2+}\) and exacerbate injury. In contrast, if NCX operates in reverse mode to bring Ca\(^{2+}\) into the myocyte, then NCX inhibitors should be cardioprotective. Inhibitors of NCX are under development as therapies to reduce ischemic injury.\(^8\)\(\sim\)\(^10\) and it is important to clearly demonstrate whether inhibition is beneficial. However, the role and direction of NCX during ischemia is debated, as is the relative role of NCX during ischemia versus reperfusion.\(^7\) The lack of selective inhibitors for NCX have made it difficult to directly examine the role and direction of NCX during ischemia and reperfusion.\(^11\) Experiments with mice that overexpress NCX\(^12\)\(\sim\)\(^13\) show increased ischemia/reperfusion injury, and the recent development of mice with cardiac-specific ablation of NCX provides a unique opportunity to evaluate the role of NCX in ischemia/reperfusion injury.

Much of the data suggesting a role for Ca\(^{2+}\) during ischemia arise from studies showing that inhibition of Na\(^+\)/H\(^+\) exchange (NHE) is cardioprotective and blocks the rise in Na\(^+\) and Ca\(^{2+}\) during ischemia.\(^5\)\(\sim\)\(^14\) The protection afforded by NHE inhibition has generally been attributed to inhibition of the rise in Ca\(^{2+}\), but there are studies suggesting that inhibition of the rise in Na\(^+\) may also contribute to cardioprotection, independent of the effect of Na\(^+\) on Ca\(^{2+}\).\(^15\)\(\sim\)\(^16\) Because NHE inhibitors block both the rise in Ca\(^{2+}\) and Na\(^+\), they cannot be used to distinguish the relative protective effects of attenuating the rise in Na\(^+\) versus Ca\(^{2+}\). Ablation of NCX would not block Na\(^+\) entry via NHE and...
thus should be useful in distinguishing the beneficial effect of attenuating Na\(^+\) versus Ca\(^{2+}\) overload.

Mice with cardiac-specific ablation (exon 11 was deleted from the NCX gene) of NCX (NCX-KO) were developed using Cre/loxP technology.\(^{17}\) In these mice, \(\approx 80\%\) to \(90\%\) of the myocytes expressed no NCX. These mice survive to adulthood, and myocytes have normal Ca\(^{2+}\) transients.\(^{17}\) Baseline contractility in NCX-KO hearts was modestly \((\approx 20\%\) decreased without any change in high-energy phosphate content, but \(\beta\)-adrenergic stimulation increased contractility to the same extent in NCX-KO hearts as in wild-type (WT) hearts. Using these mice, we examined the role of NCX in ischemia-reperfusion injury. Consistent with reverse-mode NCX activity during ischemia, we found that the rate of decline in ATP was reduced during ischemia, ischemic contracture was delayed, and maximum contracture was reduced. The NCX-KO hearts were significantly protected against ischemia-reperfusion injury using a number of indices. NCX-KO hearts had less necrosis, better recovery of contractile function, improved phosphocreatine (PCr) recovery, and reduced Na\(^+\) overload during reperfusion.

### Materials and Methods

#### Isolated Perfused Mouse Hearts

Cardiac-specific NCX knockout (NCX-KO) mice created with the Cre/loxP system of gene ablation (exon 11), described previously,\(^{17}\) and their wild-type littermates were used in this study. Mice were 8 to 9 weeks of age at the time of experimentation. All animals were treated in accordance with the National Institutes of Health Publication No. 85-23: Guide for the Care and Use of Laboratory Animals.

Hearts were isolated and perfused in the Langendorff mode as described previously.\(^{18}\) Hearts were perfused with modified Krebs–Henseleit buffer containing (in mmol/L) 120 NaCl, 25 NaHCO\(_3\), 5.9 KCl, 1.2 MgSO\(_4\), 1.75 CaCl\(_2\), and 10 glucose, gassed with 95% O\(_2\)/5% CO\(_2\) at 37°C (pH 7.4). After a 20-minute stabilization period, hearts were subjected to 20 minutes of zero-flow global ischemia followed by 120 minutes of reperfusion. Left-ventricular developed pressure (LVDP), LV peak minus end-diastolic pressure [EDP], and the rate pressure product (RPP) were determined by measuring the fluorescence intensity of drops of internal solution containing fura-2 via the pipette solution. [Ca\(^{2+}\)]i was calculated from the ratio \((R)\) of the fluorescent intensities at the two excitation wavelengths (ratios at 600 Hz) using the method of Grynkiewicz et al\(^{20}\) according to the equation:

\[
[\text{Ca}^{2+}]_i = K_{d_{\text{Ca}}} \beta (R - R_{\text{max}}) / (R_{\text{max}} - R)
\]

\(R_{\text{max}}, R_{\text{min}}, \) and \(\beta\) were determined by measuring the fluorescence intensity of drops of internal solution containing fura-2 and either high or low Ca\(^{2+}\); \(K_{d_{\text{Ca}}}=224 \text{ mmol/L}\).

As previously reported, 10% to 20% of myocytes from NCX-KO hearts have a WT phenotype; they show an NCX-specific inward current \((i_{\text{NCX}})\) during rapid exposure (1 s) of caffeine. We excluded the myocytes with a detectable \(i_{\text{NCX}}\) from the NCX-KO group.

#### 31P- and 23Na-NMR Measurement of Energy Metabolites and [Na\(^+\)]

Relative changes in the concentration of ATP, PCr, and intracellular pH (pHi) were determined by measuring ATP and reperfusion by acquiring consecutive 5-minute 31P-NMR spectra using a Varian 500 MHz spectrometer with an 11.7 Tesla superconducting magnet at the 31P resonance frequency of 202.47 MHz. The pHi was calculated from the chemical shift between inorganic phosphate (P\(_i\)) and PCr from 31P-NMR spectra as described previously.\(^{19}\) Intracellular Na\(^+\) concentration ([Na\(^+\)]i) was measured by consecutive 2.5 minutes 23Na-NMR spectra at the Na\(^+\) resonance frequency of 132.4 MHz. For Na\(^+\) measurement, 5 mmol/L of the paramagnetic shift reagent TmDOTP\(^+\) (thulium[III]1,4,7,10-tetra-azacyclo-dodecane-1,4,7,10-tetra[methylene-phosphonic acid]), sodium salt) was introduced to separate the extracellular Na\(^+\) peak from the intracellular Na\(^+\) signal. Additional CaCl\(_2\) was added to the perfusate to compensate for binding of Ca\(^{2+}\) to the TmDOTP\(^+\). Relative changes (expressed as a percent of the initial value) were reported.\(^{21}\)

### Statistical Analysis

Results are expressed as mean±SEM. Significance \((P<0.05)\) was determined by unpaired \(t\) test (for discrete variables) and repeated-measures ANOVA.

### Results

#### Animal Phenotype and Basal Contractile Performance

The Table shows basal contractile function in the NCX-KO hearts and their WT littermates. The heart weight was not significantly different between WT \((0.13±0.01 \text{ g})\) and NCX-KO \((0.14±0.01 \text{ g})\). As shown in the Table, left-ventricular developed pressure (LVDP), +dP/dt\(_{\text{max}}\), and −dP/dt\(_{\text{min}}\) were significantly lower in NCX-KO hearts than WT. The HR was not significantly different between WT and NCX-KO hearts \((P>0.05)\). These findings are consistent with the recently published echocardiographic data using an in vivo model.\(^{17}\)
Effects of NCX Ablation During Ischemia/Reperfusion

WT and NCX-KO mice were subjected to 20-minute zero-flow global ischemia at 37°C (n=10/group). As shown in Figure 1A, the time to ischemic contracture in NCX-KO hearts was significantly delayed (17.0±1.23 minutes) compared with WT hearts (10.2±1.0 minutes, P<0.001). Also, the maximum contracture or end-diastolic pressure during ischemia was significantly lower in NCX-KO (36±5 cmH2O) than in WT hearts (76±5 cmH2O, P<0.0001), consistent with reduced Ca2+ entry during ischemia. On reperfusion, NCX-KO hearts showed less postischemic contractile dysfunction than WT hearts. The postischemic recovery of LVDP (expressed as a percentage of initial preischemic LVDP) was significantly better in NCX-KO (46±4%, P<0.0001) than in WT hearts (12±2%). Postischemic recovery of rate pressure product (RPP=LVDP×HR) was also significantly better in NCX-KO than in WT hearts (P<0.0001), as shown in Figure 1B and the Table. In addition, infarct size was significantly smaller in NCX-KO hearts (7.0±2.5% of total ventricle) than in WT hearts (49.5±8.7%, P<0.01; Figure 1C). These results are consistent with the hypothesis that reverse-mode NCX activity contributes to ischemia/reperfusion injury.

Reverse-Mode NCX Is Absent in NCX-KO Myocytes

To confirm the absence of reverse-mode NCX in NCX-KO myocytes, we performed the experiment shown in Figure 2. NCX reverse mode generates the influx of 1 Ca2+ ion into the myocyte in exchange for 3 Na+ ions being extruded. Because of the electrogenic nature of this process, NCX reverse mode is favored by depolarization of the myocyte. It also depends on the Na+ gradient and thus the extracellular Na+ concentration. To provoke reverse exchange, we depolarized patch clamped myocytes from a holding potential of -40 mV to +80 mV for 1 s while simultaneously removing Na+ from the extracellular solution using a rapid solution exchanger. In 5 WT cells, this led to an increase in [Ca2+], to an average peak of 486±79 nmol/L. In contrast, none of the 5 NCX-KO myocytes exhibited an increase in [Ca2+], using this protocol.

Contractile Performance

<table>
<thead>
<tr>
<th></th>
<th>LVP Peak (cmH2O)</th>
<th>EDP (cmH2O)</th>
<th>LVDP (cmH2O)</th>
<th>HR (bpm)</th>
<th>+dP/dtmax (cmH2O/s)</th>
<th>−dP/dtmin (cmH2O/s)</th>
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<tr>
<td>Basal condition</td>
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<tr>
<td>WT</td>
<td>114±4</td>
<td>11±3</td>
<td>103±4</td>
<td>369±11</td>
<td>3513±243</td>
<td>−3272±174</td>
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<tr>
<td>NCX-KO</td>
<td>99±2</td>
<td>15±2</td>
<td>84±2*</td>
<td>365±15</td>
<td>2640±177*</td>
<td>−2259±133*</td>
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<td>At 40 minutes of reperfusion</td>
<td></td>
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<tr>
<td>WT</td>
<td>32±4</td>
<td>20±4</td>
<td>12±2</td>
<td>231±22</td>
<td>498±113</td>
<td>−294±63</td>
</tr>
<tr>
<td>NCX-KO</td>
<td>61±4*</td>
<td>22±2</td>
<td>39±4*</td>
<td>271±20</td>
<td>1367±187*</td>
<td>−988±143*</td>
</tr>
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n=10 per group. *P<0.05 vs WT.

Figure 1. LVDP traces before and during ischemia/reperfusion (A), %RPP after reperfusion (B), the cross-sectional images of WT and NCX-KO hearts incubated with 2,3,5-triphenyltetrazolium chloride (C), and infarct size measured at 120 minutes of reperfusion (D). *P<0.05 vs WT.

Figure 2. NCX reverse mode is absent in isolated cardiac KO myocytes. Myocytes were patch clamped and dialyzed with the Ca2+ indicator fura-2. Cells were held at -40 mV and were depolarized to +80 mV for 1 s. Simultaneously, myocytes were rapidly exposed to Na+ -free external solution. The tracings are representative of 5 WT and 5 KO myocytes.
Effects of NCX Ablation on Energy Metabolism and $[\text{Na}^+]_i$ Regulation

To explore further the mechanism of the protection observed in NCX knockout hearts, we measured changes in $[\text{PCr}]$, $[\text{ATP}]$, pHi, and $[\text{Na}^+]_i$ before and during ischemia/reperfusion. $^{31}$P-NMR spectroscopy was used to measure high-energy phosphates and pHi. As shown in Figure 3, at baseline, there were no differences in high-energy phosphates between WT and NCX-KO hearts. However, during ischemia, the rate of decline in ATP was slower in NCX-KO hearts than WT hearts ($P<0.05$). Furthermore, the recovery of ATP and PCr during reperfusion was significantly better in NCX-KO hearts than in WT hearts ($P<0.05$; Figure 4A and 4B). The slower fall in $[\text{ATP}]$ during ischemia in NCX-KO hearts is consistent with the delay in ischemic contracture in NCX-KO hearts and may also be related to reduced $\text{Ca}^{2+}$ accumulation. Basal pHi was similar between WT (7.22±0.03) and NCX-KO hearts (7.22±0.02; $P=0.05$), and pH decreased similarly during ischemia. At the start of reperfusion, pH recovered more rapidly in NCX-KO than WT hearts (Figure 4C), consistent with better preservation of high-energy phosphates. Taken together, these data indicate that NCX-KO have reduced ischemia/reperfusion injury.

We also examined $[\text{Na}^+]_i$ using $^{23}$Na-NMR and the shift reagent TmDOTP. There were no differences in $[\text{Na}^+]_i$ at baseline in WT hearts (14.4±0.4 mmol/L) compared with NCX-KO hearts (14.5±0.5 mmol/L; $n=5$/group, $P=0.51$). As shown in Figure 5, during 20 minutes of ischemia, $[\text{Na}^+]_i$ increased to 189% of the preischemic value in WT hearts (open circles). This increase was slightly, but significantly higher than that observed in NCX-KO hearts (162±7%, $P<0.05$; closed circles). On reperfusion, $[\text{Na}^+]_i$ recovered more completely in NCX-KO hearts than WT. After 40 minutes of reperfusion, $[\text{Na}^+]_i$ was significantly lower in NCX-KO hearts (92±3% of preischemic) than in WT hearts (128±5%).

Effects of NCX Ablation During Ischemia After Isoproterenol Addition to Normalize Workload

Because the NCX-KO hearts have a 20% reduction in contractility at baseline, it is possible that the reduced workload at the start of ischemia may contribute to the reduced ischemic injury observed in the NCX-KO hearts. We therefore briefly treated hearts with 5 nmol/L isoproterenol for 5 minutes just before ischemia to normalize the workload at the start of ischemia. Treatment with isoproterenol resulted in a similar rate-pressure product in NCX-KO and WT hearts before ischemia, as shown in Figure 6A. The changes in PCr and ATP after addition of isoproterenol were also similar between WT and NCX-KO hearts (Figure 6B). Interestingly, even with workload normalized between genotypes, NCX-KO hearts exhibited reduced ischemia/reperfusion dysfunction during ischemia. As shown in Figure 6C, rate of decline in ATP was slower in NCX-KO hearts than WT hearts ($P<0.05$). Furthermore, the time to ischemic contracture was reduced in isoproterenol-treated NCX-KO hearts compared with WT hearts (WT: 10.3±0.8 minutes; NCX-KO: 15.9±2.4 minutes), as was the maximum ischemic contracture (WT: 71±5 cmH$_2$O; NCX-KO: 36±12 cmH$_2$O; $P<0.05$). NCX-KO hearts also showed better recovery of contractile function after reperfusion (Figure 6D; $P<0.05$).

Discussion

NCX1 is ablated in 80% to 90% of the cardiomyocytes in the NCX-KO hearts. These mice live to adulthood and exhibit...
Role of NCX in Ischemia/Reperfusion Injury

Attenuating the rise in \( \text{Na}^+ \) during ischemia has been shown to decrease the rise in \( \text{Ca}^{2+} \) during ischemia. This has been taken as support for a role of NCX in the rise in [\( \text{Ca}^{2+} \)].\(^4,6\) However, NCX operates near equilibrium, and it is unclear whether the beneficial \( \text{Na}^+ \)-dependent effects on \( \text{Ca}^{2+} \) during ischemia are attributable to the inhibition of \( \text{Ca}^{2+} \) efflux (via forward-mode NCX) or reduced \( \text{Ca}^{2+} \) influx (via reverse-mode NCX).  

As inhibition of NCX is a current target for drug development to reduce ischemic injury, it is important to determine whether NCX operates in the forward mode or reverse mode during ischemia. Inhibition of exchange would have opposite effects in the 2 cases. The availability of mice with cardiac-specific ablation of NCX in 80% to 90% of the myocytes provides an ideal opportunity to directly evaluate the role of NCX in ischemia/reperfusion injury. Figure 2 clearly demonstrates that reverse-mode \( \text{Ca}^{2+} \) influx into the myocyte is absent in NCX-KO mice. Our data further show that ablation of NCX results in a significant improvement in postischemic function and significantly less myocyte death. NCX-KO hearts also exhibit less of a decline in ATP during ischemia and significantly better recovery of ATP and PCr on reperfusion. This improvement in postischemic function and reduced rate of decline in ATP during ischemia was still observed in isoproterenol-treated NCX-KO hearts, indicating that the protection observed in NCX-KO hearts is maintained when workload at the start of ischemia is normalized. Taken together, these data clearly show that lack of NCX is beneficial during ischemia. Ablation of NCX would not benefit if NCX primarily extruded \( \text{Ca}^{2+} \) from the cell (ie, net \( \text{Ca}^{2+} \) efflux) during ischemia. These data support the hypothesis that inhibition of \( \text{Ca}^{2+} \) entry via reverse-mode NCX is protective.

NCX During Ischemia Versus Reperfusion

Intracellular \( \text{Na}^+ \) rises during ischemia, as shown in this article and in several previously published studies.\(^6,21,23,24\) Because of the marked decrease in pH during ischemia, it has generally been assumed that NCX becomes inhibited.\(^25\) Although NCX activity is reduced at low pH, it is not totally inhibited, particularly under conditions of elevated \( [\text{Na}^+] \).\(^26,27\) The increase in [\( \text{Ca}^{2+} \)] during ischemia has been shown to be modulated by intracellular \( \text{Na}^+ \), suggesting that NCX operates during ischemia.\(^4\) Data in this article also indicate that NCX is active during ischemia. If NCX were totally inhibited during ischemia, then one would not expect differences during ischemia between NCX-KO and WT hearts. During ischemia we observed a slower decline in ATP, a slower rate of ischemic contracture, a reduced maximum contracture, and less of a rise in intracellular \( \text{Na}^+ \) in NCX-KO hearts. Taken together, these data suggest increased \( \text{Ca}^{2+} \) entry via NCX during ischemia in WT hearts. The lack of \( \text{Ca}^{2+} \) entry via the reverse-mode NCX would decrease the detrimental effects of \( \text{Ca}^{2+} \) overload and better preserve ATP, which would prolong the activity of the \( \text{Na}^+/\text{K}^+ \)-ATPase.\(^28,29\) leading to less \( \text{Na}^+ \) accumulation during ischemia in NCX-KO hearts. On reperfusion, the better-preserved ATP in the NCX-KO hearts facilitates \( \text{Na}^+ \) extrusion by \( \text{Na}^+/\text{K}^+ \)-ATPase.\(^28–30\)
Role of Na\(^+\) Versus Ca\(^{2+}\) in Ischemia/Reperfusion Injury

Attenuating the rise in \([\text{Ca}^{2+}]\), during ischemia using NHE inhibitors has been shown to reduce ischemia/reperfusion injury, suggesting a causal role for elevated \([\text{Ca}^{2+}]\) in the genesis of irreversible myocyte injury.\(^{31}\) However, inhibition of NHE reduces the rise in \(\text{Na}^+\) as well as the rise in \(\text{Ca}^{2+}\) during ischemia. The rise in \(\text{Na}^+\) has been suggested to have detrimental effects that occur independent of the effects on \(\text{Ca}^{2+}\).\(^{15,16}\) With inhibition of NHE, we previously reported a complete block in the rise in \(\text{Na}^+\) during ischemia,\(^{4}\) whereas in the current study we find that loss of NCX causes only 10% reduction in the rise in \(\text{Na}^+\) during ischemia. Despite these differences in \(\text{Na}^+\) during ischemia, the protection was similar (an \(\approx\)3-fold improvement in recovery of LVDP) with NHE inhibitors compared with NCX-KO hearts. Taken together, these data suggest that a rise in \(\text{Na}^+\) per se during ischemia is not a major determinant of ischemia/reperfusion injury.\(^{4}\)

In summary, hearts from mice lacking NCX have significantly less ischemia/reperfusion injury than WT hearts when subjected to a global model of ischemia. NCX-KO hearts have less necrosis, improved postischemic LVDP, higher levels of high-energy phosphates, and improved recovery of ion homeostasis. These data suggest that inhibition of NCX is a promising target for cardiac protection.

Acknowledgments

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