Mice With a Null Mutation in the NHE1 Na\(^+\)-H\(^+\) Exchanger Are Resistant to Cardiac Ischemia-Reperfusion Injury

Yigang Wang, Jamie W. Meyer, Muhammad Ashraf, Gary E. Shull

Abstract—Pharmacological studies indicate that Na\(^+\)-H\(^+\) exchanger isoform 1 (NHE1) plays a central role in myocardial ischemia-reperfusion injury; however, confirmation by alternative methods is lacking. To address this issue, we examined the role of NHE1 in ischemia-reperfusion injury using gene-targeted NHE1-null mutant (Nhe1\(^{-/-}\)) mice. Nhe1\(^{-/-}\) and wild-type hearts were perfused in a Langendorff apparatus in both the absence and presence of the NHE1 inhibitor eniporide, subjected to 40 minutes of ischemia and 30 minutes of reperfusion, and the effects of genetic ablation or inhibition of NHE1 on hemodynamic, biochemical, and pathological changes were assessed. In the absence of eniporide, left ventricular developed pressure, end-diastolic pressure, and coronary flow were significantly less impaired in Nhe1\(^{-/-}\) hearts relative to wild-type hearts, and release of lactate dehydrogenase, morphological damage, and ATP depletion were also significantly less. In the presence of eniporide however, wild-type hearts were significantly protected and there were no significant differences between the two genotypes with respect to cardiac performance, lactate dehydrogenase release, or morphological damage. Furthermore, the presence or absence of eniporide had no apparent effect on the degree of cardioprotection observed in Nhe1\(^{-/-}\) hearts. These data demonstrate that genetic ablation of NHE1 protects the heart against ischemia-reperfusion injury. In addition to providing direct evidence that confirms previous pharmacological studies indicating a role for NHE1 in ischemia-reperfusion injury, these results suggest that the long-term absence of NHE1 does not elicit major compensatory changes that might negate the cardioprotective effects of blocking its activity over the short-term. (Circ Res. 2003;93:776-782.)

Key Words: Na\(^+\)-H\(^+\) exchange ■ Sle9a1 ■ cariporide ■ ischemia ■ reperfusion

Beginning with the work of Karmazyn,\(^1\) it has become apparent that treatment with amiloride and related compounds exerts a strong protective effect against cardiac ischemia-reperfusion (I/R) injury. In the initial study,\(^1\) it was suggested that the likely pharmacological target was the sarcolemmal Na\(^+\)-H\(^+\) exchanger. Cloning studies have demonstrated the existence of five plasma membrane and three intracellular Na\(^+\)-H\(^+\) exchangers in mammalian tissues.\(^2,3\) However, among the plasma membrane isoforms only NHE1 is expressed at significant levels in heart.\(^4-7\) The hypothesis that inhibition of NHE1 is responsible for amiloride-mediated protection against I/R injury was strengthened by later studies using amiloride derivatives with greater specificity for NHE1 than for the other isoforms.\(^8-10\) In addition to amiloride derivatives, some closely related compounds that inhibit NHE1, including several with high therapeutic potential, have been shown to have protective effects in the mammalian heart.\(^11-18\)

The mechanism by which these compounds protect the heart from I/R injury is thought to be a reduction in NHE1-mediated Na\(^+\)-H\(^+\) exchange, which reduces the rate of recovery of intracellular pH and the accumulation of Na\(^+\) within the cardiac myocyte during reperfusion.\(^3,19\) There is evidence that the reduction in cytosolic Na\(^+\) reduces Ca\(^{2+}\) overload and subsequent hypercontracture that occurs via inhibition or reversal of the Na\(^+\)-Ca\(^{2+}\) exchanger,\(^3,20-22\) and that hypercontracture is further reduced by the continuing cytosolic acidification.\(^23-25\) Despite experimental support for this mechanism, amiloride and related compounds also affect other Na\(^+\) transport proteins, including Na\(^+\) channels\(^26\) and the Na\(^+\)-Ca\(^{2+}\) exchanger.\(^27\) Although it is unlikely that a significant portion of the cardioprotective effects of NHE inhibitors might be due to interactions with receptors other than NHE1, this possibility has not been rigorously excluded. For example, low concentrations of 5-(N-ethyl-N-isopropyl)-amiloride (EIPA) and HOE694 inhibit the NBC1 (also termed NBC3) Na\(^+\)-HCO\(_3^\)-cotransporter,\(^26,28\) NBC1 is one of at least four Na\(^+\)-HCO\(_3^\)-cotransporters in the heart,\(^28,30-33\) none of which have been well characterized with respect to their pharmacological profiles. Like NHE1, the Na\(^+\)-HCO\(_3^\)-cotransporters mediate Na\(^+\)-dependent alkalinization.\(^34,35\) Inhibition of NBC1 appears to provide protection against I/R injury.\(^36-38\)

In the present study, we used a combined genetic and pharmacological approach to assess the hypothesis that blocking the activity of NHE1 protects against cardiac I/R...
injury. Given the use of NHE1 inhibitors in clinical trials for a number of cardiovascular diseases, it is important to determine, by some means other than pharmacological inhibition, that ablation of NHE1 activity is cardioprotective. To accomplish this, we performed ischemia-reperfusion studies of the isolated perfused heart using a mouse model in which the Nhe1 gene (gene locus symbol Slc9a1) was disrupted. These studies were performed in the absence and presence of eniporide, an NHE1 inhibitor, to assess the possibility that the drug has additional effects other than inhibition of NHE1. Our data provide direct evidence that the loss of NHE1 protects the heart against I/R injury and indicate that the cardioprotective effects of NHE inhibitors are not due to interactions with receptors other than NHE1.

Materials and Methods

Mice and Genotype Analysis

Male and female wild-type (WT) and null mutant (Nhe1−/−) mice generated by gene targeting at the University of Cincinnati were maintained on a mixed background of 129/SvJ and Black Swiss strains. Genotypes were determined by PCR analysis of tail DNA. These experiments were approved by the University of Cincinnati Animal Care and Use Committee.

Langendorff Heart Preparation and Measurements of Cardiac Function

Hearts from Nhe1−/− and WT mice were cannulated and retrogradely perfused at 37°C and 80 mm Hg with Krebs-Henseleit buffer (118 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L MgSO4, 1.2 mmol/L KH2PO4, 2.5 mmol/L CaCl2, 25 mmol/L NaHCO3, 0.5 mmol/L Na-EDTA, and 11 mmol/L glucose, saturated with 95% O2-5% CO2, pH 7.4) through the aorta in a noncirculating Langendorff apparatus as described. A water-filled balloon was inserted into the left ventricle and adjusted to a left ventricular end-diastolic pressure (LVEDP) of 5 to 8 mm Hg. The distal end of the catheter was connected to a Digi-Med Heart Performance Analyzer via a pressure transducer (Case).

Hearts were paced at 350 bpm except during ischemia, and pacing was reinitiated after 3 minutes of reperfusion. After a 25-minute equilibration period, hearts were subjected to 40 minutes of no-flow normothermic global ischemia, followed by 3 minutes of reperfusion. Experiments were performed in the absence and presence of the NHE1 inhibitor eniporide (kindly provided by Garrett Gross, Medical College of Wisconsin) at a concentration of 23 nmol/L, which is 5 to 30 times the reported EC50.18,42 As normal controls (nonischemic), six wild-type and four Nhe1−/− hearts were perfused for 95 minutes. In the experiments performed in the absence of eniporide, hearts from six Nhe1−/− mice and six WT mice were used to assess cardiac function during ischemia and reperfusion and for measurement of lactate dehydrogenase (LDH) and ATP; an additional four hearts each from Nhe1−/− and WT mice were subjected to ischemia and used for ATP measurements. In the experiments performed in the presence of eniporide, hearts from four Nhe1−/− and WT pairs were used to assess cardiac function during ischemia and reperfusion and for measurement of lactate dehydrogenase.

Measurement of Lactate Dehydrogenase and ATP

LDH in the coronary effluent during reperfusion was determined by a coupled-enzyme spectrometric technique using a Sigma assay kit as described. ATP was extracted from 20 to 80 mg of freeze-dried left ventricle collected at the end of ischemia or after termination of the experiment and analyzed by spectrophotometry.

Morphological Analysis

Tissue from the left ventricular free wall was fixed with 2.5% buffered glutaraldehyde. A semiquantitative estimate of cell damage was performed as described previously, with ~500 cells analyzed for each heart.

Statistical Analysis

All values are expressed as mean±SEM. Group comparisons were performed using a Bonferroni/Dunn test. Individual values were compared using a students t test.

Results

Baseline Values for Cardiovascular Function

The baseline data for Nhe1−/− and WT mice used in the ischemia-reperfusion studies performed in the absence or presence of eniporide are shown in Table 1. Mean body weight and heart weight of Nhe1−/− mice were both significantly lower than those of WT mice (P<0.05). There was no significant difference between the mean heart weight/body weight ratios of Nhe1−/− mice and WT mice in either the untreated or eniporide-treated groups (0.78% and 0.75%, respectively), suggesting that the smaller hearts in the knockout were due to growth retardation. Basal preischemic left ventricular developed pressure, left ventricular end-diastolic pressure (which was set at the beginning of the experiment), and coronary flow did not differ significantly between the two genotypes.

Cardiac Function During Ischemia and Reperfusion in the Absence of an NHE1 Inhibitor

The magnitude and the time course of ischemic contracture development for Nhe1−/− and WT hearts in the absence of eniporide, assessed by the progressive increase in LVEDP, are shown in Figure 1A. Beginning at 20 minutes of ischemia, Nhe1−/− hearts exhibited a reduction in the degree of ischemic contracture when compared with WT hearts. The maximum level of contracture, observed at 30 minutes of ischemia, was
Effects of NHE1 ablation on cardiac function during ischemia and reperfusion.

Figure 1. Effects of NHE1 ablation on cardiac function during ischemia and reperfusion. Nhe1−/− and WT hearts were retrogradely perfused in a Langendorff apparatus and then subjected to no-flow ischemia, followed by reperfusion. Hearts were paced at 350 bpm during the initial equilibration period. Pacing was terminated during ischemia and reinitiated at 3 minutes into the reperfusion period. As normal controls, WT hearts were perfused and paced throughout the 95-minute period. A, Left ventricular end-diastolic pressure (LVEDP) during ischemia and reperfusion was lower in Nhe1−/− than in WT hearts. B, Left ventricular end-diastolic pressure (LVEDP) during ischemia and reperfusion was lower in Nhe1−/− than in WT hearts. C, Coronary flow during reperfusion was higher in Nhe1−/− hearts than in WT hearts subjected to I/R injury. Values are mean ± SEM; n=6 hearts for each group; *P<0.05 vs WT control.

There were major differences between Nhe1−/− and WT hearts in postischemic recovery of cardiac function as judged by attenuation of the elevation in LVEDP that occurred during the 30-minute reperfusion period (Figure 1A). In Nhe1−/− hearts, LVEDP was significantly lower than that of WT hearts at the beginning of reperfusion and decreased steadily throughout the reperfusion period. In contrast, WT hearts exhibited an initial increase in LVEDP at 3 minutes of reperfusion (when pacing of the heart was reinitiated), which was followed by a gradual decrease. At the end of the reperfusion period, LVEDP was significantly lower in Nhe1−/− hearts (25.4±14.5 mm Hg) than in WT hearts (60.7±9.9 mm Hg).

On the basis of left ventricular developed pressure (LVDP), recovery of postischemic contractile function was greater in Nhe1−/− hearts than in WT hearts (Figure 1B). Preischemic LVDP was ≈110 mm Hg in both WT and Nhe1−/− hearts and dropped to 0 mm Hg during the 40-minute period of ischemia. LVDP in the normal controls was also ≈110 mm Hg at the beginning of the experiment and dropped to ≈90 mm Hg by the end of the 95-minute experimental protocol. Within 3 minutes of the beginning of reperfusion, LVDP rose to 45±2.3 mm Hg in the knockout, but to only 20.0±8.2 mm Hg in WT hearts. By the end of the 30-minute reperfusion period, LVDP was 55.6±13.6 mm Hg in the Nhe1−/− group and 31.3±10.2 mm Hg in the WT group. Thus, a significantly greater functional recovery was observed in Nhe1−/− hearts (50.5% of preischemic value) compared with WT hearts (27.7% of preischemic value).

During reperfusion, Nhe1−/− hearts exhibited a better recovery of coronary flow than WT hearts (Figure 1C). Before ischemia, coronary flow was ≈12.5 mL/min per gram heart weight and increased in both experimental groups during the first 3 minutes of reperfusion (Figure 1C). However, the coronary flow rate during reperfusion was ≈30% to 35% higher in Nhe1−/− hearts than in WT hearts.

Cardiac Function During Ischemia and Reperfusion in the Presence of an NHE1 Inhibitor

If the cardioprotective effects of NHE1 inhibitors were due entirely to their effects on NHE1, then one would predict that treatment of Nhe1−/− hearts to an inhibitor would lead to no additional cardioprotection. Also, if the cardioprotection occurring in untreated Nhe1−/− hearts as a result of NHE1 ablation is attenuated by changes that are secondary to the long-term absence of NHE1, such as activation of alternative Na+-dependent acid extrusion mechanisms, then the degree of I/R injury should differ between Nhe1−/− and WT hearts treated with an NHE1 inhibitor.

To examine these issues, isolated hearts were exposed to the NHE1 inhibitor eniporide throughout the equilibration and ischemia-reperfusion periods. There were no significant differences in LVEDP, LVDP, or coronary flow between hearts for the two genotypes when treated with inhibitor (Figure 2), nor were there any significant differences when these values were compared with those of Nhe1−/− hearts (Figure 1) that were not treated with inhibitor. However, the
values for both genotypes were significantly different from the values for untreated WT hearts subjected to I/R injury and from the normal WT and Nhe1−/− controls (not subjected to the I/R protocol) (Figure 2). The Nhe1−/− normal control (nonischemic) exhibited a slightly greater reduction in LVDP than the WT control after 95 minutes of perfusion; however, the differences were not statistically significant.

**Release of LDH and ATP Content**

The accumulated amount of LDH released during 30 minutes of reperfusion after global ischemia is shown in Figure 3. LDH released from WT hearts treated with eniporide (5.8 ± 0.3 U/g) or from Nhe1−/− hearts in either the absence (5.6 ± 0.8 U/g) or presence (5.2 ± 0.5 U/g) of eniporide were significantly less (P < 0.001) than that of WT hearts that were not treated with eniporide (13.2 ± 1.4 U/g). In the studies performed in the absence of eniporide, left ventricular ATP content (Figure 4) was significantly higher (P < 0.001) in the Nhe1−/− hearts than in WT hearts both at the end of ischemia (8.6 ± 0.9 and 4.25 ± 0.7 μmol/g dry wt, respectively) and at the end of reperfusion (10.3 ± 1.2 and 5.3 ± 0.9 μmol/g dry wt, respectively).

**Pathological Changes**

The degree of cell damage resulting from ischemia and reperfusion is shown in Table 2 and Figure 5. In normal WT (Figure 5A) and Nhe1−/− (Figure 5D) control hearts that were not subjected to ischemia, cellular structure was well preserved. In WT hearts subjected to ischemia and reperfusion in the absence of eniporide, the percentage of normal, mildly damaged, and severely damaged cells was 13.6 ± 3.2, 14.2 ± 1.2, and 72.2 ± 4.2, respectively (Table 2); many myofibers in the WT hearts exhibited evidence of hypercontraction injury and release of intracellular contents, and intracellular vacuolization was frequently observed (Figure 5B). In contrast, the ischemia-reperfusion protocol caused significantly less cellular damage in the corresponding Nhe1−/− hearts (Figure 5C) and in both WT (Figure 5E) and Nhe1−/− (Figure 5F) hearts treated with eniporide, where myocytes...
knockouts die before weaning, apparently as a result of epileptic seizures, and additional mice die after weaning. On some occasions, simply handling an adult knockout mouse before an experiment was sufficient to bring on a seizure and sudden death. On the basis of the similarity between the responses of Nhe1−/− and WT hearts treated with eniporide, it seems unlikely that the susceptibility to seizures and growth retardation influences the response of Nhe1−/− hearts to ischemia or that the surviving Nhe1−/− mice used for our experiments represented a subset of mice that were more resistant to stress. Also, the heart weight/body weight ratios were essentially the same in both genotypes, suggesting that the absence of NHE1, by itself, has no significant effect on growth of the heart. Despite the difficulties in working with Nhe1−/− mice, it was possible to obtain sufficient numbers of adult animals to conduct these experiments. Nevertheless, the problems in using the NHE1 knockout are formidable, and long-term studies of the role of NHE1 in cardiac function, I/R injury, and ischemic preconditioning would be greatly facilitated by the development of a tissue-specific knockout.

The smaller rise in LVEDP in Nhe1−/− hearts (Figure 1) and the better preservation of ATP levels at the end of ischemia (Figure 4) demonstrate that genetic ablation of the Nhe1 gene exerts cardioprotective effects during ischemia. The cardioprotective effects of NHE1 ablation were also evident during the reperfusion period, in which the mutant hearts exhibited lower LVEDP, higher LVDP, and reduced leakage of LDH. Furthermore, at the end of the reperfusion period, cellular structure and ATP levels were restored better in Nhe1−/− hearts. These results are virtually identical to those of studies in which isolated rabbit12 or guinea pig14 hearts were treated with the NHE1 inhibitors HOE694 or cariporide (HOE642). A comparison of the

Table 2. Semiquantitative Estimate of Morphological Damage in Hearts From Nhe1−/− and WT Mice Subjected to Ischemia-Reperfusion

<table>
<thead>
<tr>
<th>Group (n=4)</th>
<th>Degree of Cell Damage, Percent of Cells</th>
<th>Zero (Normal)</th>
<th>Mild</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control (WT)</td>
<td>96.8±0.6</td>
<td>2.4±0.5</td>
<td>0.8±0.2</td>
<td></td>
</tr>
<tr>
<td>Normal control (Nhe1−/−)</td>
<td>97.1±0.4</td>
<td>2.3±0.7</td>
<td>0.6±0.2</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>13.6±0.2</td>
<td>14.2±1.2</td>
<td>72.2±1.0</td>
<td></td>
</tr>
<tr>
<td>Nhe1−/−</td>
<td>46.2±1.3*</td>
<td>25.5±0.6*</td>
<td>28.3±1.7*</td>
<td></td>
</tr>
<tr>
<td>Nhe1−/−+EN</td>
<td>38.3±2.3*</td>
<td>37.8±3.9*</td>
<td>24.0±1.8*</td>
<td></td>
</tr>
<tr>
<td>WT+EN</td>
<td>39.5±2.1*</td>
<td>29.3±2.4*</td>
<td>31.2±1.0*</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean±SEM; *P<0.01 vs wild type. Nhe1−/− indicates NHE1 knockout; EN, eniporide.
effects of NHE1 ablation with the effects of inhibition by eniporide revealed no further effects on Nhe1−/− hearts, suggesting that NHE1 alone is the pharmacological receptor. These comparisons also showed that the cardioprotective effect of NHE1 inhibition in WT hearts was not significantly different from that observed in Nhe1−/− hearts. Thus, it is now clear from both pharmacological and genetic studies that loss of NHE1 activity protects against cardiac I/R injury.

Coronary flow during reperfusion was reduced in WT hearts (Figure 1C), relative to that in Nhe1−/− hearts or hearts of either genotype treated with eniporide (Figure 2C). This finding suggests that the activity of NHE1 in the cardiac myocyte confers a greater susceptibility to no reflow ischemia during reperfusion. Because this phenomenon arises as a result of damage occurring during ischemia, it provides further evidence that either genetic ablation or pharmacological inhibition of NHE1 provides protection during ischemia. Also, because no reflow ischemia during reperfusion can lead to further damage, it clouds the issue of how much of the cardioprotective effect of NHE1 ablation or inhibition is occurring during ischemia.

The better preservation of ATP levels at the end of ischemia (Figure 4) and other indications of cardioprotection afforded by NHE1 ablation during ischemia are not entirely consistent with the original proposal that I/R injury is primarily the result of increased Na+ entry during reperfusion, via the Na+-H+ exchanger, and subsequent increases in intracellular Ca2+. More recent studies have shown that intracellular Na+ also rises during ischemia, and that this rise is at least partially dependent on Na+-H+ exchange.20-22 If NHE1 activity is genetically ablated or inhibited during ischemia, then this should lead to both a more rapid acidification, which reduces contractile activity,24,44 and a reduction in the amount of Na+ to be extruded by the Na+-K+-ATPase. This would be expected to reduce not only the consumption of ATP during ischemia, but also hypercontracture-induced injury and subsequent no reflow ischemia during reperfusion.

Most of the experimental studies with NHE inhibitors have examined the cardioprotective effects of acute pharmacological exposures, and some of the potential therapeutic uses of these inhibitors would involve such short-term treatments. However, recent studies have shown that longer-term treatment with NHE1 inhibitors might protect against cardiac hypertrophy and heart failure.47,48 One concern about long-term therapy using these inhibitors is the possibility that compensation might occur that would lessen the therapeutic benefits. The apparent upregulation of NHE1 in response to treatment of normal rats with NHE inhibitors raises the possibility that cardiac injury might be enhanced if myocardial infarction were to occur just after the inhibitor was withdrawn.49 On the other hand, cariporide reduced NHE1 expression and hypertrophy in a transgenic mouse model in which the β1-adrenergic receptor was overexpressed in heart.48 This suggests that alterations in NHE1 levels might not be a problem in certain disease states and that, in fact, an NHE1 inhibitor might block remodeling that would lead to upregulation of NHE expression as a secondary effect. With regard to the possibility of other alterations affecting the therapeutic potential of NHE1 inhibitors, the results of the current study, in which genetic ablation or inhibition of NHE1 had similar cardioprotective effects, demonstrates that the long-term absence of NHE1 does not elicit secondary changes that negate the direct cardioprotective effects of the absence of NHE1 activity.

In summary, the experiments presented in this study provide direct, nonpharmacological evidence that eliminating NHE1 activity is cardioprotective during ischemia and reperfusion and that genetic ablation of NHE1 does not elicit long-term remodeling that blunts the cardioprotective effects. Further studies will be needed to determine whether the loss of NHE1 alters responses to ischemic preconditioning and to assess the effects of NHE1 ablation during performance in vivo.

Acknowledgments

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