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Overexpression of the Na^+/H^+ exchanger and ischemia-reperfusion injury in the myocardium

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Imahashi K, Mraiche F, Steenbergen C, Murphy E, Fliegel L. Overexpression of the Na^+/H^+ exchanger and ischemia-reperfusion injury in the myocardium. *Am J Physiol Heart Circ Physiol* 292: H2237–H2247, 2007. First published January 5, 2007; doi:10.1152/ajpheart.00855.2006.—In the myocardium, the Na^+/H^+ exchanger isoform-1 (NHE1) activity is detrimental during ischemia-reperfusion (I/R) injury, causing increased intracellular Na^+ (Na_i^+) accumulation that results in subsequent Ca^{2+} overload. We tested the hypothesis that increased expression of NHE1 would accentuate myocardial I/R injury. Transgenic mice were created that increased the Na^+/H^+ exchanger activity specifically in the myocardium. Intact hearts from transgenic mice at 10–15 wk of age showed no change in heart performance, resting intracellular pH (pH_i) or phosphocreatine/ATP levels. Transgenic and wild-type (WT) hearts were subjected to 20 min of ischemia followed by 40 min of reperfusion. Surprisingly, the percent recovery of rate-pressure product (%RPP) after I/R improved in NHE1-overexpressing hearts (64 ± 5% vs. 41 ± 5% in WT; $P < 0.05$). In addition, NMR spectroscopy revealed that NHE1 overexpressor hearts contained higher ATP during early reperfusion (levels $P < 0.05$), and there was no difference in Na^+ accumulation during I/R between transgenic and WT hearts. HOE642 (cariporide), an NHE1 inhibitor, equivalently protected both WT and NHE1-overexpressing hearts. When hearts were perfused with bicarbonate-free HEPES buffer to eliminate the contribution of HCO_3^- transporters to pH_i regulation, there was no difference in contractile recovery after reperfusion between controls and transgenics, but NHE1-overexpressing hearts showed a greater decrease in ATP during ischemia. These results indicate that the basal activity of NHE1 is not rate limiting in causing damage during I/R, therefore, increasing the level of NHE1 does not enhance injury and can have some small protective effects.

transgenic mice; sodium/hydrogen exchange; intracellular pH; intracellular sodium

THE NA^+/H^+ EXCHANGER (NHE) is a ubiquitously expressed plasma membrane glycoprotein that extrudes one intracellular proton in exchange for one extracellular sodium. It thereby protects cells from intracellular acidification while facilitating extracellular Na^+ entry into the cytosol (12). Nine isoforms of NHE have been discovered and are designated NHE1-NHE9. NHE1 was the first isoform discovered (35) and is ubiquitously expressed in the plasma membrane of mammalian cells. Other isoforms have more restricted tissue distributions and some have predominantly intracellular localization. In mammals, NHE1 plays a key role in regulation of cell pH, cell volume, and cell proliferation (12).

Na^+/H^+ exchange has been suggested to play a critical role in intracellular Ca^{2+} overload during ischemia-reperfusion (1, 27, 48). During ischemia-reperfusion, NHE1 activity results in increases in intracellular Na^+, which results in Na^+ accumulation during ischemia and early reperfusion when the Na^+-K^+-ATPase is inhibited. This increase in intracellular Na^+ leads to an increase in intracellular Ca^{2+} via the Na^+/Ca^{2+} exchanger leading to Ca^{2+} overload and subsequent contractile dysfunction (3, 27, 31, 46). This hypothesis is supported by many studies showing that NHE1 inhibitors, including EIPA, cariporide, eniporide, and zoniporide, prevent or reduce ischemia-reperfusion injury in various models (3, 9, 23, 31, 46). A recent study using mice null for NHE1 showed that hearts from these mice had less ATP depletion during ischemia and reperfusion and improved recovery of contractile function after reperfusion (45). However, despite the wealth of data suggesting that inhibition of NHE1 results in reduced ischemia-reperfusion injury, several recent clinical trials, including guard during ischemia against necrosis (GUARDIAN trial), evaluation of the safety and cardioprotective effects of eniporide in acute myocardial infarction (ESCAMI trial), and EXPEDITION show varying results (2, 34, 48). The ESCAMI trial (48) showed that the NHE1 inhibitor eniporide did not limit infarct size or clinical outcome. However, in the GUARDIAN trial (42), a subset of patients undergoing high-risk coronary artery bypass graft surgery showed significant improvement with cariporide administration. Similarly, the Na^+/H^+ Exchange Inhibition to Prevent Coronary Events in Acute Cardiac Conditions study showed an improvement in the incidence of myocardial infarction with cariporide administration to humans; however, this was offset by an increase in cerebrovascular events (29).

In addition to its role in ischemia-reperfusion injury, there is increased NHE1 activity in hypertensive, hypertrophied, or diabetic myocardium (22). NHE1 activity has also been shown to be elevated in human patients with end-stage heart failure (47), in isolated cardiomyocytes subjected to chronic acidosis (10), and in hearts subjected to ischemia (13). Furthermore, protein kinases that regulate NHE1 activity are activated in the myocardium by ischemia and reperfusion (30). The elevation of NHE1 activity in the myocardium in disease states, plus its role in ischemia-reperfusion injury, suggests that increased NHE1 activity could be maladaptive to the myocardium. To evaluate the effect of overexpression of NHE1 on basal cardiac function as well as on ischemia-reperfusion injury, we created

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transgenic mice that overexpress NHE1 in the myocardium. We studied cardiac function in isolated hearts perfused with HCO_3^--containing buffer and HCO_3^--free HEPES buffer. Our results showed that NHE1 overexpression had no significant effect on baseline cardiac function, energy metabolism, or intracellular pH (pH_i) in both types of buffer. Interestingly, NHE1 overexpression did not enhance ischemia-reperfusion injury and did not result in differences in pH_i or intracellular Na^+ during ischemia-reperfusion.

MATERIALS AND METHODS

Construction of NHE1-overexpressing mice. Two types of transgenic mice were created. One line (N-line) contained the wild type (WT) NHE1 overexpressed in the mouse myocardium. The α-myosin heavy chain (MHC) promoter directed cardiac overexpression. The hemagglutinin (HA)-tagged NHE1 from the plasmid pYN4+ was described earlier (32). This vector was digested with *Sma*I and *Nde*I to yield a 2551 bp piece that contained the human NHE1 isoform of the NHE and an HA tag. The *Nde*I site was blunted with Klenow enzyme. A plasmid containing the 5.5-kb mouse cardiac α-MHC promoter was cut with *Sal*I enzyme that digests immediately downstream of the promoter. The site was filled in with Klenow enzyme, and the NHE1 fragment was ligated into this site. After the correct orientation was confirmed, linear DNA was excised from the plasmid using the enzyme *Not*I. Linearized α-MHC-NHE1-HA (Fig. 1*A*) was microinjected into fertilized oocytes that were transferred into the oviduct of pseudopregnant FVB/N mice. Transgenic mice were identified by PCR analysis of tail genomic DNA using a forward primer corresponding to the 3′ end of the α-MHC promoter sequence (MHCNHEf 5′-GCCCAGCTGCCCGGCACTCTTAG-3′) and a reverse primer corresponding to the 5′ end of the NHE1 cDNA sequence (MHCNHEr 5′-GCCCCACCAAAGCAACCACCAC-3′). Founder mice were identified, bred with wild-type FVB/N, and maintained in a pathogen-free environment.

A second transgenic mouse line (K-line) was made that had a mutation in the cytoplasmic regulatory domain of the protein. The mutation 1K3R4E changes amino acids Lys641, Arg643, Arg645, and Arg647 to Glu residues. This mutation has earlier been shown to cause a defect in calmodulin regulation of NHE1 (5). It results in an NHE1 protein that is alkaline shifted in its pH dependence and therefore is more active than the control at a given pH. Site-specific mutagenesis was carried out using forward and reverse synthetic oligonucleotides of the sequence, 5′-CGCAAAATCCTGAGGAACAACTTGCAGGAGACCGAGCAGGAGCTCGAGTCCTACAACAGACACACGCTGG-3′). The mutation introduced overlapping *Sac*I and *Xho*I sites that facilitated identification of correct mutants.

Protein preparation, SDS-PAGE, and Western immunoblotting. Proteins were prepared from mouse tissues, including heart, liver, kidney, lungs, and brain. Tissues were placed in a buffer containing 120 mM NaCl, 10 mM Tris (pH 7.4), 0.1 mM PMSF, 0.1 mM benzamidine, 37.5 μM ALLN (calpain I inhibitor), and a proteinase inhibitor cocktail (33) for homogenization. Samples were homogenized at 4°C for 30 s, incubated on ice for 30 s, and then homogenized again for 30 s by using an Omni International 2000 electric homogenizer. To obtain crude membrane fractions (which contained the NHE1 protein), homogenates were subjected to a series of centrifugation steps. Initial centrifugation was for 10 min at 3,000 rpm (735 *g*). The pellet was discarded and the supernatant centrifuged at 10,000 rpm for 15 min (8,200 *g*). The resulting pellet was again discarded, and the supernatant was centrifuged at 30,000 rpm (40,000 *g*) for 1 h to obtain a fraction enriched in crude microsomes. The pellet containing the membrane fraction was resuspended in the same buffer as described above with the addition of 1% SDS to aid in solubilization. Total protein was quantified using the Bio-Rad D_C Protein Assay

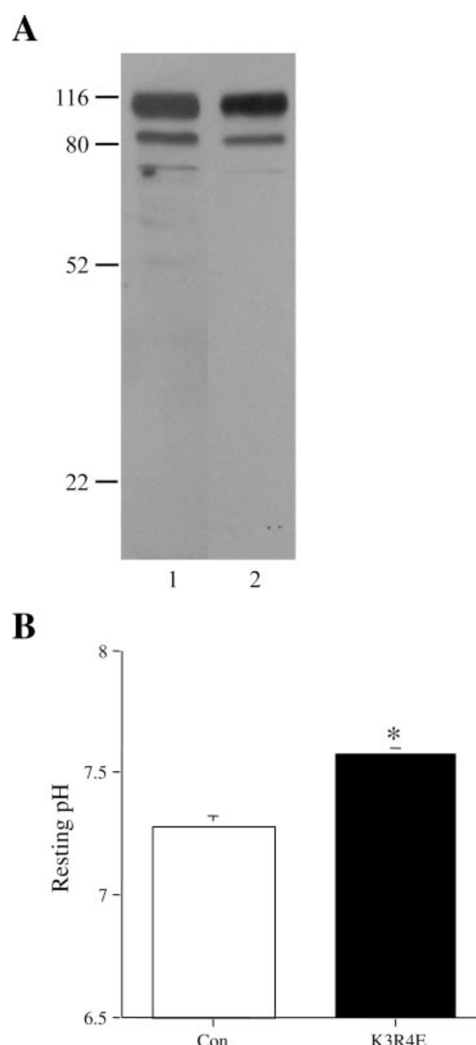

Fig. 1. Characterization of Na^+/H^+ exchanger (NHE) transgenes. *A*: Western blot of hemagglutinin (HA)-tagged NHE1 transgenes in AP-1 cells. *Lane 1*, cell extracts from stable cell line from AP-1 cells transfected with wild-type (WT) NHE1. *Lane 2*, cell extracts from stable cell line from AP-1 cells cells transfected with Lys641, Arg643, Arg645 ,and Arg647 mutated to 4 glutamic acids (1K3R4E). *B*: resting intracellular pH (pH_i) in stably transfected cell lines. AP-1 cells were transfected with either WT NHE1 (Con) or NHE1 with the 1K3R4E. Resting pH_i was measured as described earlier (32). *Significantly different from the controls, $*P < 0.05$. Results are displayed as mean ± SE of >8 values.

kit. Western blot analysis was with rabbit polyclonal anti-HA tag (Santa Cruz SC805 Y-11) used at 1:2,000 in 1% milk Tris-buffered saline as described earlier (32). In some experiments we determined the levels of membrane proteins present in samples of WT or transgenic hearts. Antibodies against Na^+-K^+-ATPase (α) was from Chemicon, for the Na^+/Ca^{2+} exchanger the polyclonal antibody was from Dr. J. Lytton (Univ. of Calgary), polyclonal anti-sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA) antibodies were from Dr. M. Michalak (Dept. of Biochemistry Univ. of Alberta), anti Na-bicarbonate cotransporter antibody type 1 isoform was from Chemicon, while polyclonal anti-AE3 and AE1 antibodies were from Dr. J. Casey (Dept. of Physiology, of the University of Alberta).

Cell culture and stable transfection. AP-1 cells that lack endogenous NHE activity were maintained in α-MEM. For transfection, 4×10^5 cells were seeded in a 35-mm Petri dish in 2 ml of growth media. The following day, cells were transfected with 4 μg of WT or

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mutagenized plasmid construct using Lipofectamine 2000 Reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. Transfected cells were selected by using 800 μg/ml geneticin (G418) in α-MEM. Stable cultures were regularly reestablished from frozen stocks, and cells from passage numbers in the range 5–15 were used for experiments (32). pH_i was measured in AP-1 cells as described earlier (32).

Isolated perfused mouse heart. The hearts of male mice were excised and perfused in Langendorff mode as described previously (20). The perfusion buffer was Krebs-Henseleit 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 (pH = 7.4 at 37°C). To eliminate other pH regulatory mechanisms such as bicarbonate exchanger, some hearts were perfused with bicarbonate-free HEPES buffer containing (in mmol/l) 128 NaCl, 5.9 KCl, 1.2 $MgCl_2$, 5 HEPES, 1.75 $CaCl_2$, and 10 glucose, gassed with 100% O_2 (pH = 7.4 by addition of NaOH at 37°C). After a 20-min stabilization period, hearts were subjected to 20 min of global zero-flow ischemia followed by 40 min of reperfusion. Left ventricular (LV) developed pressure (LVDP = LV peak pressure − end-diastolic pressure), heart rate (HR), rate of contraction (+dP/d*t*), and rate of relaxation (−dP/d*t*) were monitored via a fluid-filled balloon in the left ventricle connected to a pressure transducer. After ischemia, the recovery of function was estimated by using the percent recovery of the rate-pressure product (RPP) (i.e., LVDP × HR) normalized to the preischemic value. In some hearts, an NHE inhibitor (1 μM), HOE642, also known as cariporide (36), was administered at 10 min before ischemia and treated through ischemia until 20 min of reperfusion.

Measurement of [ATP], [PCr], pH_i, and intracellular Na^+. Relative changes in the concentration of ATP, PCr, and pH_i were measured during ischemia-reperfusion by acquiring 5 min ^{31}P NMR spectra using a Varian 500-MHz NMR spectrometer with an 11.7-Tesla superconducting magnet at the ^{31}P resonance frequency of 202.47 MHz. pH_i was calculated using the shift difference between the inorganic phosphate and PCr peaks (20). To measure intracellular Na^+ concentration, hearts were perfused with a paramagnetic shift reagent thulium[III]-tetra-azacyclo-dodecane-1,4,7,10-tetra(methylene-phosphonic acid), sodium salt ($TmDOTP^{5-}$) (20, 21). The frequency for ^{23}Na was 132.4 MHz, and 2.5-min ^{23}Na-NMR spectra were acquired during ischemia followed by 15 min of reperfusion. The heart in the NMR tube was maintained at 37°C using variable temperature system.

pH_i measurement in isolated cardiomyocytes. Adult ventricular myocytes were isolated from WT and transgenic mice by collagenase digestion (16). In brief, mice were heparinized and then anesthetized using euthanyl. The hearts were excised and placed in ice-cold Ca^{2+}-free perfusion [containing (in mM) 113 NaCl, 4.7 KCl, 0.6 KH_2PO_4, 0.6 Na_2HPO_4, 1.2 $MgSO_4 \cdot 7H_2O$, 12 $NaHCO_3$, 12 $KHCO_3$, 20 D-glucose, 10 Na^+-HEPES, 2 creatine, 2 carnitine, 30 taurine, 10 mM 2,3-butanedione monoxime; pH 7.2, at 37°C]. Non-cardiac tissue was quickly removed, and the heart was cannulated via the aorta. The heart was then perfused with continuously gassed (95% O_2-5% CO_2) Ca^{2+}-free perfusion buffer for 4 min at 37°C, followed by perfusion with enzymatic digestion buffer [containing Ca^{2+}-free perfusion buffer, 0.08% collagenase (Type II Worthington), 25 μM Ca^{2+}, and 0.1% BSA, continuously equilibrated with 95% O_2-5% CO_2 at 37°C] for 3 min. By the end of the digestion period, the heart became soft and flaccid, and the ventricles were removed and cut into five to six pieces and placed in storage medium (containing Ca^{2+}-free perfusion buffer, 100 μM $CaCl_2$, and 0.1% bovine serum albumin). This suspension was dispersed by gentle trituration through a wide-bore disposable serological pipette. The cell suspension was maintained in storage buffer at 25°C for at least 2 h before use in the microepifluorescence studies.

pH_i was monitored in single ventricular myocytes using the pH-sensitive fluorescent dye 2,7-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) (Calbiochem). Two hours after isolation, 500 μl of cell suspension were placed on a coverslip coated with 10 μg/ml laminin (Collaborative Biomedical Products), mounted on the stage of an inverted microscope (Leica), and set at a temperature of 37°C. Cells were then loaded with 12 μM BCECF for 30 min. A single healthy cell was then used for the remaining experiment. The cells were excited with light at 490 and 440 nm, and the resulting fluorescence emission intensity from a single myocyte was measured simultaneously at 530 nm (Photon Technology International), which contained an adjustable aperture and a single photomultiplier tube. The cells were superfused (1 ml/min) with normal buffered solution [containing (in mM) 135 NaCl, 4.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 20 HEPES, and 10 glucose, adjusted to pH 7.4 with NaOH at 37°C]. After an initial measurement period of resting pH_i, cells were acidified by using 50 mM NH_4Cl as described earlier (38). The rate of recovery from acid load was measured after normal buffered solution was returned. After every measurement of pH_i, the excitation intensity ratio (490/440) was calculated and converted to a pH_i scale, using in situ calibration data obtained by exposing cells loaded with BCECF to nigericin-containing calibration solutions of pH 6.5 to 7.8.

Buffering capacity (B, $mmol \cdot l^{-1} \cdot pH\ unit^{-1}$) was determined by varying amounts of NH_4Cl and by observing the change of cell pH produced by this load. BCECF-loaded cells were incubated with NH_4-containing buffer as described earlier (37).

$$B = \Delta NH_{4i}^{+}/\Delta pH_i$$

$$(NH_4Cl_o \times 10(pK_a - pH_i))/[1 + 10(pK_a - pH_O)]$$

The equilibrium between NH_4^+, NH_3, and pH in the extracellular medium is determined by the Henderson-Hasselbach relation using a pK_a for NH_4^+ of 9.21. Proton flux (J_{H^+}) produced due to the NHE1 after acid loading was estimated as the product of the rate of pH_i change (dpH/d*t*), buffering capacity ($mmol \cdot l^{-1} \cdot pH\ unit^{-1}$).

$$(J_{H^+}) = d(B \cdot pJ)/dt$$

Buffering capacity was determined on isolated cardiomyocytes of WT and transgenic mice that were grown on coverslips for up to 48 h as described earlier (37).

Statistical analysis. Data are expressed as means ± SE. Significance was determined by repeated-measures ANOVA or unpaired *t*-test (2 groups) as appropriate.

RESULTS

Production and characterization of the 1K3R4E NHE1 mutant. For these experiments we used both a WT NHE1 and an NHE1 mutant that had the amino acids Lys641, Arg643, Arg645, and Arg647 mutated to 4 glutamic acids (1K3R4E). After the mutation was made and confirmed by DNA sequencing, we transfected the mutant and control DNA into NHE-deficient AP-1 cells. Figure 1*A* is a Western blot of two stable cells lines made by transfecting HA-tagged NHE1 into NHE-deficient AP-1 cells. *Lane 1* shows the immunoreactivity of the WT NHE1, whereas *lane 2* is that of cells transfected with the 1K3R4E mutant. Both the WT and the mutant NHE1 expressed in AP-1 cells as detected by anti-HA tag antibody. The predominant immunoreactive species is 110 kDa, whereas a more minor secondary band of size ~90–95 kDa was also found. This immunoreactive band has previously been suggested to represent unglycosylated or partially glycosylated protein (32). We measured the resting pH_i of these cells in bicarbonate-free medium after loading with fluorescent compounds as described earlier (32). The results are shown in Fig. 1*B*. Cells that had an NHE1 with the 1K3R4E mutation had an elevated resting pH_i

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of ~0.25 pH units greater than those with control NHE1. These results confirmed that the mutation was functional as described earlier by others (44).

Production and characterization of transgenic mice with NHE1 expressed in the myocardium. In transgenic mice, cardiac-specific overexpression of NHE1 was driven by the cardiac MHC promoter (Fig. 2*A*). Figure 2*B* is a Western blot using antibody against HA. *Lane 1* is an extract of a positive control made from a stable cell line with HA-tagged NHE1 transfected into NHE-deficient AP-1 cells. The predominant immunoreactive species is 110 kDa, whereas a more minor secondary band of size ~90–95 kDa was also found. Extracts were made from hearts of control and transgenic mice and these were blotted for the presence of the transgene using the HA tag as a marker. *Lanes 2* and *3* are heart extracts from nontransgenic mice, whereas *lanes 4* and *5* are extracts from hearts of transgenic mice. The transgenic mice showed strong immunoreactive bands of glycosylated and unglycosylated NHE1, whereas the control mice showed no immunoreactivity with anti-HA antibody.

Figure 2*C* is a Western blot that examines the tissue-specific expression of transgenic mice (N-line). The antibody used for this blot was anti-HA tag, so that only exogenous NHE1 is detected. The expression of the NHE1 was found in the heart extracts (*lane 3*), and there was no detectable expression in the lung, liver, kidney, or brain (*Lanes 4–7*, respectively). *Lane 1* is from a non-transgenic heart and *lane 2* is a positive control. Similar results were obtained with the K-line transgenic mice. The NHE1 was expressed almost exclusively in the heart. A trace of expression of a lower-molecular-weight immunoreactive species was sometimes seen in brain tissues (Fig. 2*C*, *lane 7*).

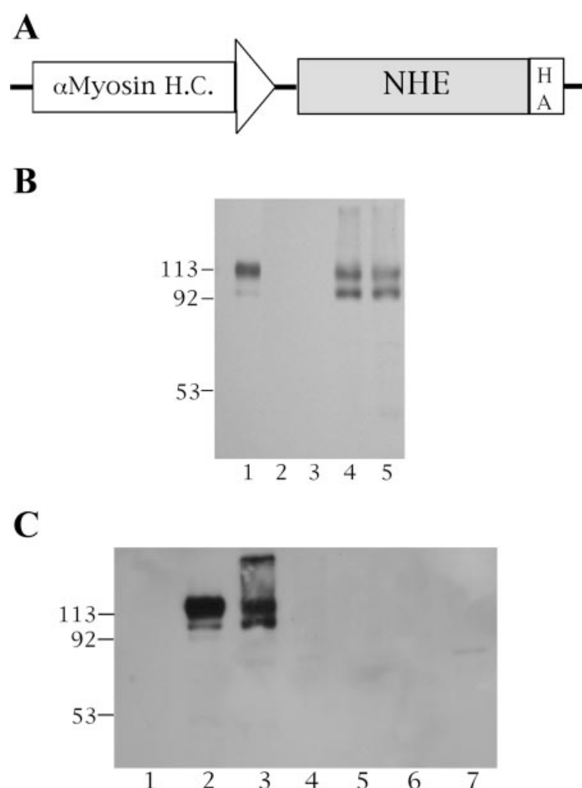

Fig. 2. Expression of NHE transgenic mice. *A*: schematic diagram of NHE1 transgenic construct. α-Myosin H.C., α-myosin heavy chain promoter. *B*: Western blot analysis of transgenic mice examined for expression of the HA-tagged NHE. *Lane 1*, positive control made by transfecting AP-1 cells with HA-tagged NHE. *Lanes 2* and *3* extracts of control non-transgenic hearts. *Lanes 4* and *5*, extracts of hearts from two independent lines of transgenic mice. *C*: tissue-specific expression of N-line mice. *Lane 1*, extract of a non-transgenic heart. *Lane 2*, positive control, cell extract from AP-1 cells transfected with HA-tagged NHE. *Lanes 3–7* are extracts from the heart, lung, liver, kidney, and brain, respectively, of N-line mice.

We measured NHE activity using freshly isolated cardiomyocytes from WT and N-line and K-line transgenic mice. Figure 3 compares the rate of recovery of internal pH and proton flux in isolated cardiomyocytes subjected to an ammonium chloride-induced acid load. The rate of recovery of pH_i from an acute acid load was increased approximately two- and threefold in the N-line and K-line mice, respectively. Buffering capacity was measured as described earlier (37), and the proton flux was determined. The mean internal pH after ammonium chloride-induced acidification was within 0.1 pH units for all groups. Proton flux of the N-line cardiomyocytes was greatly elevated compared with the controls (330 ± 37%). While proton flux was increased twofold in K-line mice, the results were very variable (199 ± 167%) and not significantly different from FVB mice. The reason for the relatively reduced flux, despite a rapid rate of change in internal pH, was that buffering capacity was reduced in the K-line mice. For the three groups FVB, N-line, K-line, the equations for buffering capacity (B, in $mmol \cdot l^{-1} \cdot pH\ unit^{-1}$) were $B = 186.1–22.5 \cdot pH_i$, $B = 279.2–35 \cdot pH_i$, and $B = 74.6–9.3 \cdot pH_i$, respectively, calculated as described earlier (37).

To determine whether expression of NHE1 in the myocardium altered expression of other regulatory membrane proteins involved in pH regulation and sodium and calcium fluxes, we determined the level of several of these proteins using Western blotting. Extracts of hearts were made and immunoblotted for the level of Na^+-K^+-ATPase, Na^+/Ca^{2+} exchanger, SERCA, Na^+-bicarbonate cotransporter isoform 1, and anion exchanger type 3 isoform (AE3). The results are shown in Fig. 4, *A* and *B*. Figure 4*A* shows one example of each type of Western blot for these proteins. Figure 4*B* shows a summary of the results of quantification of a series of Western blots for each protein done as described earlier (33). There were no significant changes in the level of any of these proteins in the transgenic hearts compared with controls. A preliminary experiment examined the levels of AE1 in the myocardium in transgenic versus controls. These levels were very low and difficult to quantify, but there were no apparent differences between transgenics and controls (not shown).

Basal contractile performance and energetics in NHE1-overexpressing hearts. As shown in Table 1, in isolated perfused hearts, basal contractile function was similar between WT and NHE1-overexpressing hearts (N-line and K-line); LVDP, heart rate, and ±dP/d*t* were not significantly different between WT and NHE1-overexpressing hearts. We also measured myocardial ATP, PCr, and pH_i using ^{31}P NMR spectroscopy. There was no alteration in PCr/ATP in transgenic hearts (Table 1). pH_i was also not significantly different between WT (7.27 ± 0.02) and NHE1 transgenic hearts (N-line: 7.23 ± 0.01, K-line: 7.29 ± 0.04). These results indicate that overexpression of either WT or mutant NHE1 does not alter energy

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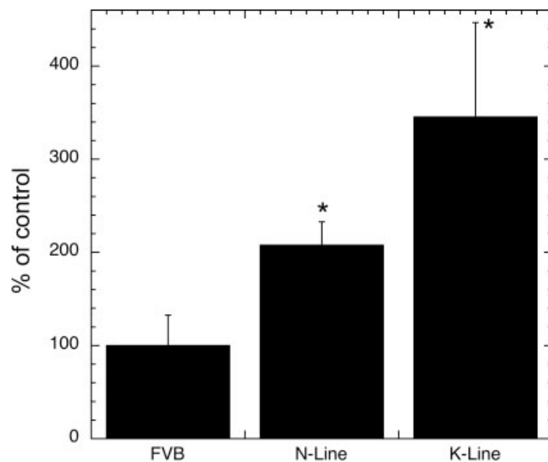
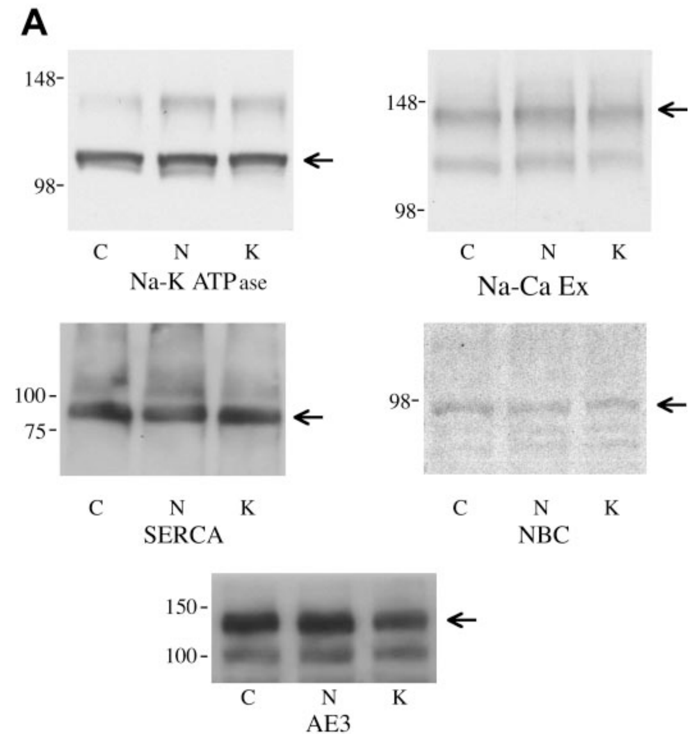


Fig. 3. NHE activity in isolated cardiomyocytes from WT, N-line, and K-line NHE1 transgenic mice. Isolated cardiomyocytes were prepared from control and transgenic mouse hearts, and freshly made cells were used to determine NHE activity as described in the MATERIALS AND METHODS. The initial rate of recovery was measured after transient induction of acid. $*P < 0.05$ vs. WT controls.

metabolism or pH_i, consistent with the lack of effects on contractile function.

Effects of NHE1 overexpression on the susceptibility to ischemia-reperfusion injury. WT and transgenic hearts were subjected to 20 min of ischemia followed by 40 min of reperfusion. We initially hypothesized that NHE1-expressing hearts would have more damage after reperfusion. However, we unexpectedly found that NHE1 overexpression improved contractile function after ischemia-reperfusion. Figure 5 illustrates the results with mice expressing the WT NHE1 isoform (N-line). Figure 5*A* shows that PCr decreased similarly during ischemia in WT and NHE1 transgenic hearts. However, the amount of ATP during the first 20 min of reperfusion was higher in transgenic hearts than that in WT hearts ($P < 0.05$, Fig. 5*B*). At the end of reperfusion, ATP levels in transgenic hearts reached the same level as WT. The ATP levels after reperfusion did not return to the preischemic values, which is typical of the results found in these studies (17). As shown in Fig. 5*C*, pH_i at the end of ischemia was not significantly different between WT (6.34 ± 0.03) and NHE1-overexpressing hearts (6.24 ± 0.08, $P = 0.26$). Interestingly, pH_i tended to recover slightly quicker in NHE1-overexpressing hearts than that in WT hearts (Fig. 5*C*). This occurred during the initial reperfusion period and is consistent with a faster recovery of ATP in NHE1-overexpressing hearts. The time to contracture during ischemia was not significantly different between WT (10 ± 1 min) and NHE1-overexpressed hearts (11 ± 1 min). We further found improved recovery of contractile function, expressed as %RPP, after reperfusion in NHE1-overexpressing hearts ($P < 0.05$ vs. WT, Fig. 5, *D* and *E*). The improvements in RPP were mostly a function of enhanced recovery of LVDP.

Effects of NHE1 overexpression on the changes in intracellular Na concentration during ischemia-reperfusion. We examined the effect of NHE1 overexpression on intracellular Na concentration at baseline, and no significant increase in intracellular Na concentration was found in the NHE1-overexpressing hearts (N-line) compared with controls (Fig. 5*F*). We also examined the effect of NHE1 overexpression on intracellular Na concentration during ischemia-reperfusion since NHE1 plays a key role in intracellular Na^+ and Ca^{2+} accumulation during ischemia and reperfusion. Using ^{23}Na NMR spectros-



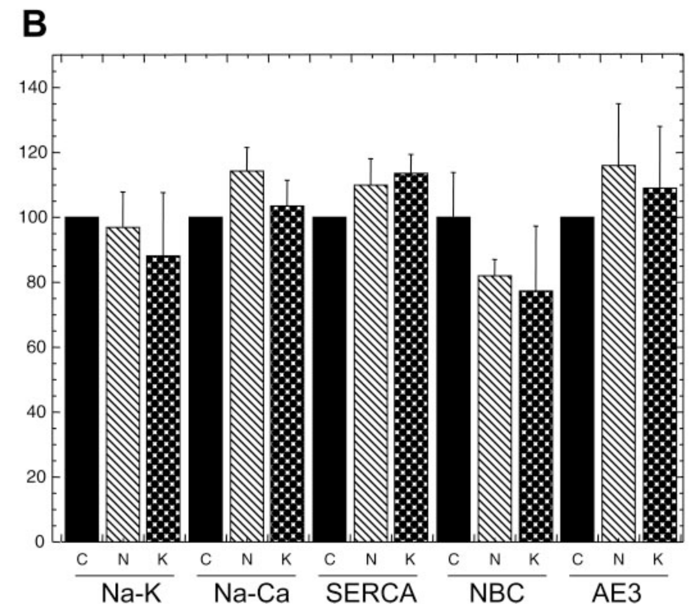


Fig. 4. Western blot analysis of expression of ion transport proteins in WT versus N-line and K-line NHE1 transgenic mice. *A*: example of Western blot with antibodies directed against Na^+-K^+-ATPase (α) (Na-K-ATP), Na^+-Ca^{2+}-exchanger (Na-Ca Ex), Sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA), Na-bicarbonate cotransporter antibody type 1 isoform (NBC), and anion exchanger type 3 (AE3). Arrows indicate the cross-reactive species of the predicted molecular weight. *B*: summary of analysis of Western blot expression of ion transport and pH regulatory proteins. C, WT control; N, N-line; K, K-line. Results are displayed as means ± SE of 4–7 values.

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Table 1. *Basal cardiac performance, PCr/ATP ratio, and intracellular pH in hearts from WT and transgenic (N-line and K-line) mice*

Group	LV_{Peak}, cmH_2O	EDP, cmH_2O	LVDP, cmH_2O	Heart Rate, beats/min	$+dP/dt_{max}$, cmH_2O/ms	$-dP/dt_{min}$ cmH_2O/ms	PCr/ATP	pH_i
Krebs-Henseleit buffer								
WT	145.2±6.2	12.7±1.6	132.5±5.5	345±21	4.9±0.2	−3.6±0.2	1.6±0.1	7.27±0.02
NHE N-line	148.6±8.2	14.4±1.4	134.2±8.1	321±17	4.7±0.4	−2.8±0.1	1.6±0.1	7.23±0.01
NHE K-line	146.6±4.1	14.0±2.6	132.5±5.7	344±8	4.8±0.2	−3.0±0.2	1.6±0.1	7.29±0.04
HEPES buffer (HCO_3^--free)								
WT	123.4±3.8	14.4±3.6	109.0±3.2	314±5	3.8±0.2	−2.4±0.2	1.8±0.2	7.20±0.08
NHE N-line	123.4±4.5	16.7±4.8	106.7±3.5	289±13	4.2±0.3	−2.6±0.2	1.8±0.2	7.18±0.08

Values are means ± SE. WT, wild-type mice; NHE, Na^+/H^+ exchanger transgenic mice; LVP_{peak}, peak left ventricular pressure; EDP, end-diastolic pressure; LVDP, left ventricular developed pressure; $\pm dp/dt_{max}$, maximal rate of contraction ($+dp/dt$) and relaxation ($-dp/dt$); pH_i, intracellular pH.

copy compared with WT hearts, we found no significant difference in intracellular Na concentration during ischemia in NHE1-overexpressing hearts (Fig. 5*F*) though there was a slight, but consistent, tendency for intracellular Na concentration to be elevated relative to WT during reperfusion. Intracellular Na concentration recovered to preischemic levels by 20 min of reperfusion in both WT and NHE1-overexpressed hearts.

Effects of NHE1 inhibition by HOE642. It has been shown that NHE1 inhibition protects the heart against ischemia-reperfusion injury. To examine the effects of NHE1 inhibition in NHE1 transgenic hearts, we treated hearts with the NHE1 inhibitor HOE642. To enhance our ability to see differences between WT and transgenic hearts, we examined transgenic hearts expressing a slightly hyperactive NHE1 (K-line). Figure 6*J* shows that HOE642 improved contractile function in both WT and NHE1-overexpressing hearts (K-line), respectively ($P <$ 0.05). Figure 5, *A–I*, illustrates the changes in PCr, pH_i, and ATP during ischemia-reperfusion. During ischemia, addition of HOE642 had no significant effect on the decline in ATP (Fig. 6, *B* and *E*) or PCr (Fig. 6, *A* and *D*) in either WT or NHE1-overexpressing hearts. In HOE642-treated WT hearts, [ATP] was higher during reperfusion than in untreated WT hearts ($P < 0.05$) (Fig. 6*B*). NHE1-overexpressing hearts treated with HOE exhibited significantly higher ATP after 40 min of reperfusion (Fig. 6*E*) compared with untreated NHE1-overexpressing hearts ($P < 0.05$). As shown in Fig. 6, *C* and *F*, NHE1 inhibition did not change pH_i significantly during ischemia both in WT and NHE1-overexpressing hearts. These results indicate that NHE1 inhibition is beneficial to both WT and NHE1 transgenic hearts. Furthermore, its overexpression is not detrimental, and again there was a slight protection conferred upon hearts that overexpressed a hyperactive form of NHE1 (Fig. 6, *G–I*).

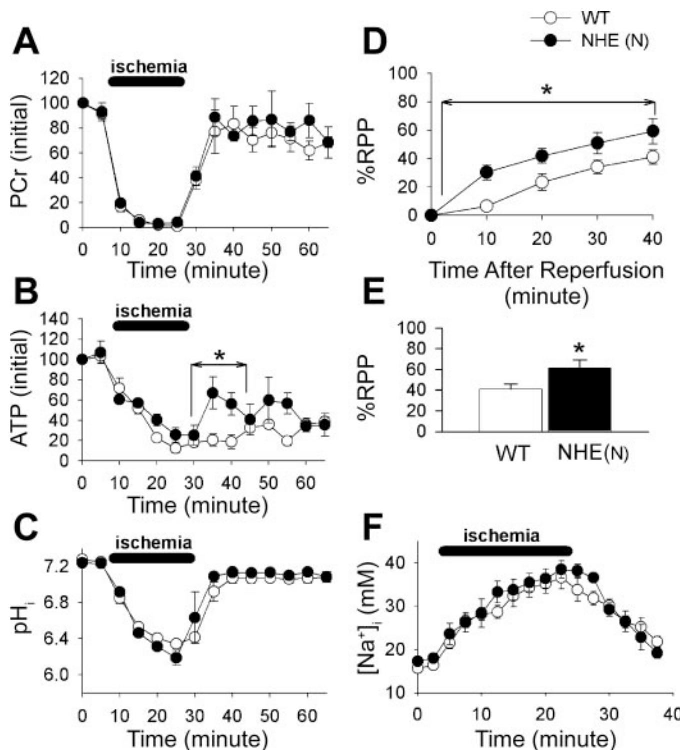

Fig. 5. Changes in high-energy phosphates, contractility, pH_i, and intracellular Na (Na_i) levels during ischemia-reperfusion in control and N-line mouse transgenic hearts perfused with Krebs-Henseleit (HCO_3^- containing) buffer. Time course of PCr (*A*), ATP (*B*), and pH_i (*C*) during ischemia-reperfusion. *D*: time course of percent rate-pressure product (%RPP) after reperfusion. *E*: %RPP at the end of ischemia. %RPP was the percent recovery of rate pressure product (left ventricular developed pressure × heart rate) normalized by preischemic value. *F*: changes in intracellular Na^+ concentration ($[Na^+]_i$) during ischemia-reperfusion.*$P < 0.05$ vs. WT controls.

Effects of NHE1 overexpression during HCO_3^--free ischemia-reperfusion. Both NHE1 and the Na^+-HCO_3^- cotransporter are stimulated by an acid load and both operate to restore pH_i following acidification. NHE1 exchanges intracellular H^+ with extracellular Na^+, whereas Na^+-HCO_3^- cotransporter transports 1 to 3 HCO_3^- and 1 Na^+ into the myocyte (25). To examine the effect of NHE1 overexpression in the absence of bicarbonate-dependent transporters, we performed studies in HCO_3^--free HEPES buffer to eliminate the role of these transporters. As shown in Table 1, under HCO_3^--free conditions in WT mouse hearts, basal pH_i was slightly more acidic than in the presence of bicarbonate (~7.3 in bicarbonate vs. ~7.2 in HEPES), but in bicarbonate-free buffer there was no significant difference in pH_i between WT (7.20 ± 0.005) and NHE1-overexpressing (N-line) hearts (7.18 ± 0.04, $P > 0.05$).

When we examined the contractile function and ATP and PCr levels in HCO_3^--free medium during ischemia and reperfusion, we found significant differences between control and transgenic (N-line) hearts (Fig. 7). During ischemia, there was an earlier significant reduction in ATP levels in NHE1 trans-

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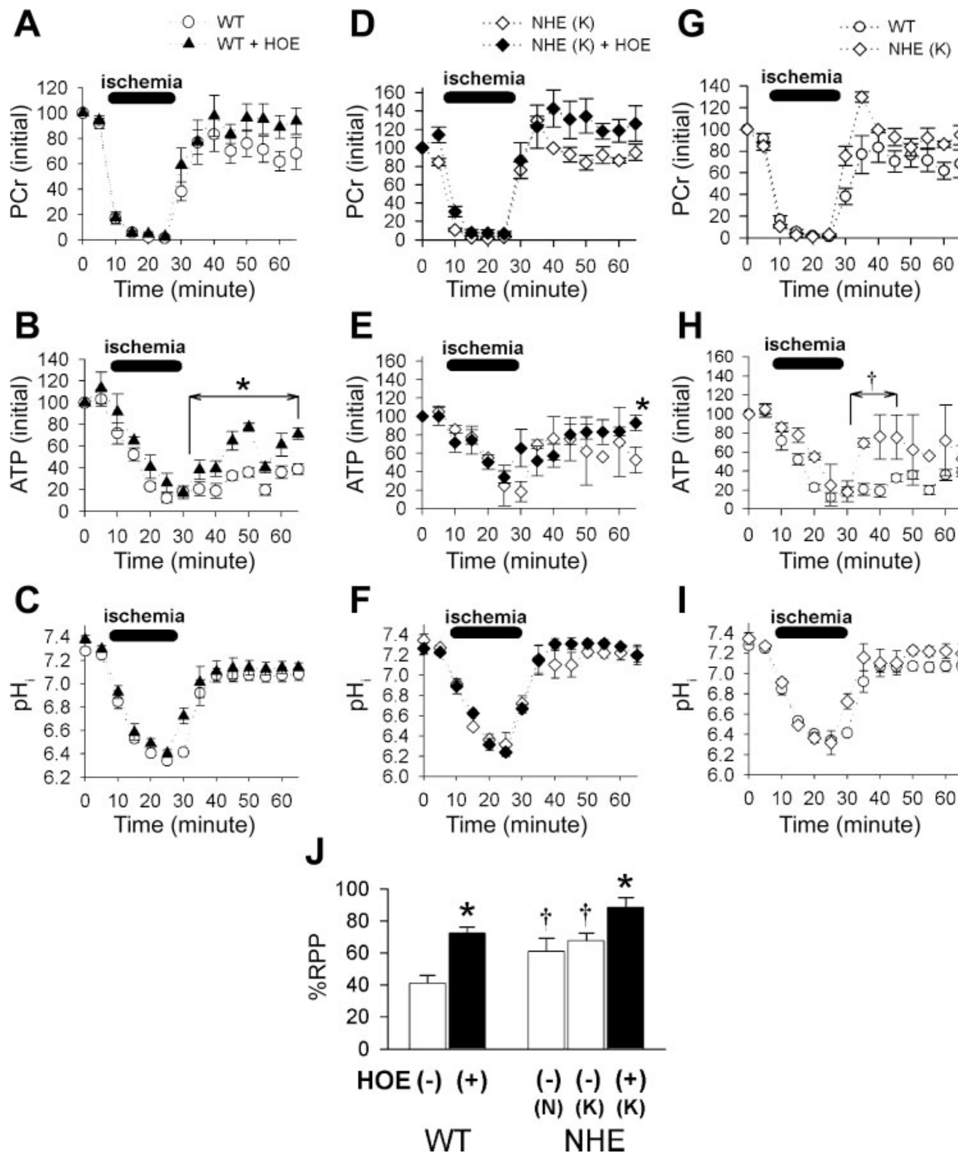

Fig. 6. Effects of NHE1 inhibition by HOE642 on changes in pH_i, contractility, and high-energy phosphates level during ischemia-reperfusion of control, N-line-, and K-line mice. Hearts were perfused with Krebs-Henseleit (HCO_3^- containing) buffer. *A–C*: WT mouse hearts (± HOE642). *D–F*: K-line (± HOE642) mouse hearts. *G–I*: comparison of WT and K-line mouse hearts. *J*: %RPP at the end of ischemia. *$P < 0.05$ vs. untreated hearts. †$P < 0.05$ vs. untreated WT hearts.

genic hearts compared with those in WT hearts ($P < 0.05$). During the first 20 min of reperfusion, NHE1-expressing hearts had lower ATP than WT hearts ($P < 0.05$) but had no difference at the end of reperfusion. There was no difference in intracellular acidification or PCr during ischemia in NHE1-expressing versus WT hearts (Fig. 7, *A–C*). Furthermore, there was no difference in recovery of function at 40 min of reperfusion between WT and NHE1-overexpressing hearts (Fig. 7, *D* and *E*).

DISCUSSION

pH_i is regulated by a number of transporters. A decrease in pH_i stimulates NHE and the Na^+-HCO_3^- cotransporter, which operate to restore pH_i to basal pH_i (~7.25). An increase in pH_i stimulates Cl-OH exchange and Cl-HCO_3^- exchange, which acid loads the cell to restore pH_i (43). Aside from steady-state pH_i maintenance, NHE activation and stimulation have also been implicated in several disease conditions, including hypertrophy and damage to the myocardium that occurs with ischemia and reperfusion (8, 11). NHE1 expression and activity have also been shown to be elevated in the mammalian and specifically in the human myocardium in several disease states (22, 47). We have also reported earlier that in the rat myocardium, ischemia followed by reperfusion activates NHE1-directed protein kinases (30) and results in an elevation of NHE1 levels (10). Thus it seems that the myocardium has several adaptive mechanisms that increase the capacity and activity of NHE1 during myocardial disease.

We therefore examined the effects of cardiac-specific overexpression of NHE1 to determine whether increased NHE1 expression and activity are maladaptive or cardioprotective. Transgenic mice were therefore created that overexpressed the NHE1 isoform of the NHE specifically in the myocardium. We used the α-MHC promoter that targeted expression specifically to the heart. Western blotting confirmed this specific myocardial expression (Fig. 2). We characterized the NHE activity in isolated cardiomyocytes from control and transgenic mice. The rate of recovery from an acute acid load was increased approx-

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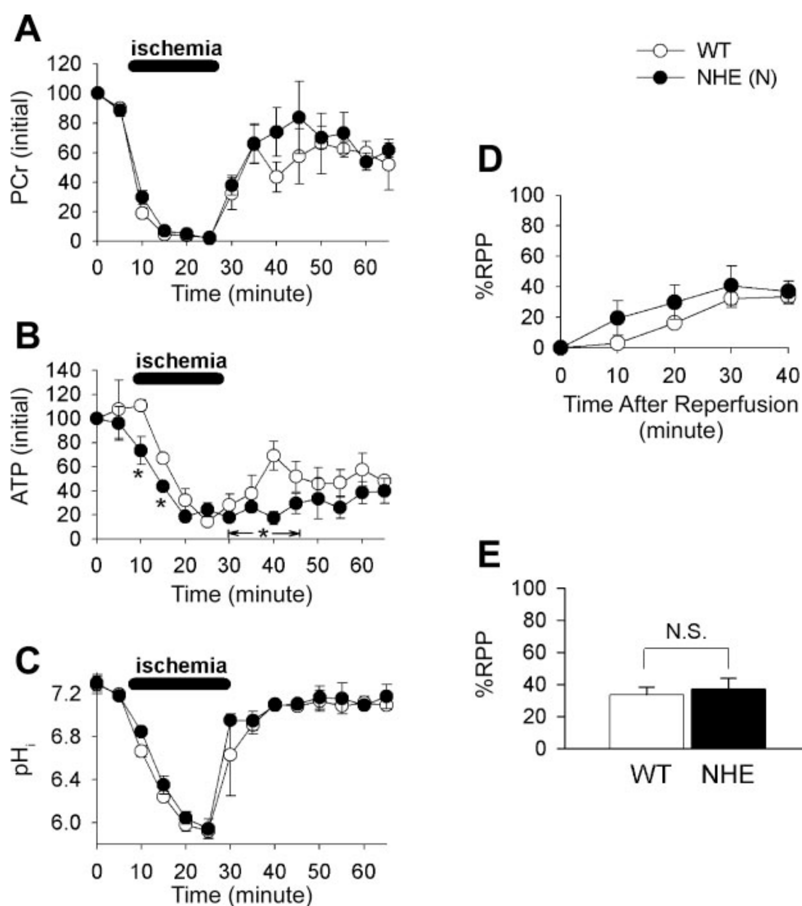

Fig. 7. Changes in PCr, contractility, and pH$_i$ during ischemia-reperfusion in control and N-line mouse hearts perfused with Krebs-Henseleit (HCO_3^--free HEPES) buffer. *A*: PCr; *B*: ATP; *C*: pH$_i$; *D*: %RPP as in Fig. 3; *E*: %RPP at the end of ischemia as in Fig. 4. *Significantly different from WT at $P < 0.05$. Asterisk with arrows indicate that NHE1-expressing transgenics were significantly different WT for the time points indicated.

imately two- and threefold in N- and K-line mice, respectively. Proton flux, directly indicative of NHE activity, was increased approximately threefold over control levels in N-line mice. Whereas proton flux was increased twofold in K-line mice, the results were very variable and not significant. The reason for the variability is not clear at this time, but we suspect that it may reflect some deterioration of the isolated cardiomyocytes during isolation, possibly reflective of the presence of the NHE1 itself. The elevation of the levels of activity that occurred in the N-line mice were similar to changes in NHE1 activity in the myocardium of humans with chronic end-stage heart failure (47).

To confirm that we did not have widespread alteration of expression of other genes or elevation of other pH regulatory proteins in the transgenic mice, we examined the levels of five ion transport membrane proteins in the transgenic hearts (Fig. 3). Of note, the pH regulatory proteins AE3 and NBC1 were not changed in the transgenics. The most abundant anion exchanger isoform present in the myocardium is the AE3 isoform (7), so we examined the levels of this protein. However, these levels were not significantly altered. The Na^{+}-bicarbonate transporter is also present in the myocardium, but its level of expression was not significantly altered. In addition, the levels of Na^{+}-K^{+}-ATPase, SERCA, and NHE were unchanged.

A comparison of basal contractile function, intracellular Na concentration, energy metabolism, and pH$_i$ showed that at 16 wk of age, hearts from transgenic mice showed no gross abnormalities. Cardiac-specific overexpression of NHE1 has no effect on basal phenotype and results in no apparent pathology in the intact mouse. It has been shown that during ischemia, Na^{+} enters via NHE1, and with depressed Na^{+}-K^{+}-ATPase activity, Na^{+} accumulates and enhances intracellular Ca^{2+} accumulation via the Na^{+}/Ca^{2+} exchanger (1, 4, 15, 19, 21, 24, 41). The resultant Ca^{2+} overload causes subsequent contractile dysfunction (14). Inhibition of NHE1 before ischemia has been shown to suppress Na^{+} accumulation (15, 18, 24, 40) and Ca^{2+} accumulation (31) during ischemia and reduce ischemia-reperfusion injury. In further support of a protective role for inhibition of NHE, a recent study showed that cardiac NHE1 null mice had reduced ischemia-reperfusion injury (45). Thus, although there are strong data suggesting that inhibition of NHE1 is protective, the effects of NHE1 overexpression are unknown. We found that NHE1 overexpression resulted in no deleterious effect on the recovery of function after global ischemia and reperfusion in NHE1-overexpressing hearts compared with WT hearts. In contrast, we found that NHE1-expressing transgenic hearts had several measures of contractile function and metabolic parameters improved compared with WT. ATP levels and %RPP were improved compared with controls. The effects were found in hearts that overexpressed WT NHE1 (N-line, Fig. 5) and in hearts that overexpressed a slightly hyperactive NHE1 (K-line, Fig. 7). The mechanism by which increased NHE1 expression improves

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some aspects of contractile performance with ischemia-reperfusion injury is not known at this time. There was a minor improvement in recovery of pH_i following ischemia and a minor improvement in recovery of ATP levels in N-line mice (Fig. 5). However, whether this could account for the improved recovery in %RPP is not clear at this time.

Addition of an NHE inhibitor HOE642 improved recovery of %RPP in both WT and NHE1-overexpressing hearts. HOE642 treatment also allowed improved recovery of ATP and PCr levels upon reperfusion in WT and NHE1-overexpressing hearts. These data are consistent with previous data showing that inhibition of NHE1 during ischemia is protective (3, 9, 15, 23, 31, 36, 41, 46). It was notable that hearts expressing the slightly hyperactive NHE (K-line) appeared to have improved recovery of ATP and PCr levels relative to control hearts. Though these improvements were at times sporadic, they nevertheless support the concept that increased activity of NHE1 is not detrimental and has some beneficial effects on the myocardium when treated with global ischemia followed by reperfusion.

Bicarbonate-dependent transporters such as Na^+-HCO_3^- cotransport play an important role in pH_i regulation (6, 28, 43). To evaluate the effects of NHE1 overexpression in the absence of the Na^+-HCO_3^- cotransporter, hearts were perfused with HCO_3^--free HEPES buffer (26). Under these conditions, pH_i regulation largely depends on the NHE1 activity in myocyte. The basal pH_i of hearts perfused with HEPES buffer was slightly but significantly more acidic compared with Krebs-Henseleit buffer. In addition, the pH_i fell more during ischemia in the hearts in bicarbonate-free buffer. Acute removal of bicarbonate has been reported to result in alkalinization of the cell, which is typically attributed to inhibition of the acid-loading Cl-bicarbonate exchanger. However, Kusuoka et al. (25) have reported that removal of bicarbonate or addition of SITS results in a decrease in pH_i in perfused ferret hearts. We found that, in the absence of bicarbonate, there was no difference in basal pH_i between WT and NHE1-overexpressing hearts, suggesting that even when HCO_3^--dependent transport is eliminated, NHE1 overexpression does not affect pH_i at basal condition. Interestingly, in the absence of bicarbonate, there was a faster rate of decline in ATP during ischemia and lower ATP during reperfusion in the NHE1-overexpressed hearts. This faster rate of decline of ATP levels in the NHE1-overexpressing hearts suggests that there may be increased energetic demands in the NHE1-overexpressing hearts. Nevertheless, this increased demand is not sufficient to alter recovery of high-energy phosphates on reperfusion or recovery of RPP.

It was significant that in the absence of bicarbonate, there was less of a beneficial effect of overexpression of NHE1 during ischemia and reperfusion. The reason for this is not yet certain but could be attributable to a number of causes. In bicarbonate-free medium, there would likely be reduced intracellular buffering in the myocyte. This might lead to more intracellular acidification and possibly more intracellular damage. It is notable that during ischemia, pH_i fell below 6.0 when hearts were perfused with bicarbonate-free medium while remaining near 6.2 in hearts perfused in the presence of bicarbonate. Greater acidification and possibly more cellular damage could have led to the reduced recovery (%RPP) compared with hearts perfused in bicarbonate-containing medium. In the absence of external bicarbonate, bicarbonate-dependent transporters could maintain an acidifying current while extruding bicarbonate produced intracellularly (39). This, together with reduced buffering capacity of the myocardium, might explain the decreased performance of transgenic hearts in bicarbonate-free medium.

We suggest that our results are not contradictory with others that have shown that inhibition of NHE1 activity or elimination of NHE1 genetically is beneficial to the myocardium during ischemia and reperfusion injury (8, 15, 22, 23, 36, 45). Whereas NHE1 inhibition is clearly beneficial in animal models, reduction of NHE1 levels during heart disease has not been adopted as an evolutionary adaptation. Rather, NHE1 expression, regulation, and activity are increased in various forms of heart disease (10, 13, 30, 47). It may be that reduction of NHE levels as a cardioprotective strategy did not evolve due to other adverse effects of reducing NHE1 levels or due to difficulties in acutely reducing gene expression to very low levels. Our results showed that while complete or near-complete NHE1 inhibition was cardioprotective, increasing NHE1 levels above endogenous ones was not maladaptive and offered some cardioprotection, at least in this experimental model. Further examination of the effect of NHE1 elevation in different types of pathologies may explain why increased NHE1 expression occurs in cardiac disease.

In conclusion, overexpression of NHE1 does not enhance susceptibility to ischemia-reperfusion injury, rather it conferred slight protection to the myocardium. This result suggests that the normal adaptive mechanisms of the myocardium–to increase NHE1 expression and activity–are not maladaptive. The mechanism whereby this cardioprotection is conferred is not yet clear. Our data also suggest that the basal activity of NHE1 is not rate limiting in causing the damage that occurs during global zero-flow ischemia of isolated perfused hearts, and therefore increasing the level or activity of NHE1 does not enhance injury through Ca^{2+} overload in this model. Whereas our experiments showed improved myocardial performance in a whole heart model of ischemia and reperfusion, it is not known whether this would also occur for other models of heart disease such as coronary artery ligation or metabolic models of ischemia. Future experiments will examine these possibilities.

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