Diastolic calcium is elevated in metabolic recovery of cardiomyocytes expressing elevated levels of the Na^+/H^+ exchanger

István Baczkó, Fatima Mraiche, Peter E. Light, and Larry Fliegel

Abstract: In the myocardium, the Na^+/H^+ exchanger isoform 1 (NHE1) plays a pivotal role in mediating ischemia–reperfusion (I/R) injury by causing intracellular Na^+ accumulation that results in a subsequent increase in intracellular calcium (Ca^{2+} overload). One of the major clinical correlates of I/R injury is contractile dysfunction, in which Ca^{2+} overload via increased Na^+/Ca^{2+} exchange is a major contributor. To better understand the cellular role of NHE1 during I/R injury, contractile function and calcium transients were measured during metabolic inhibition and recovery in single ventricular myocytes from transgenic mice with elevated NHE1 expression. During normoxic conditions, no differences were seen between NHE1-overexpressing cardiomyocytes and wild-type (WT) cardiomyocytes with respect to fractional cell shortening (FCS), rate of shortening (+dL/dt), and rate of relaxation (–dL/dt). When metabolic recovery followed metabolic inhibition, NHE1-overexpressing ventricular myocytes exhibited a significant increase in FCS (130.2% ± 11.77% baseline) and ±dL/dt (146.93% ± 12.27% baseline). This correlated with a significant increase in the concentration of diastolic intracellular calcium, which was attenuated by the NHE1 inhibitor HOE694. These results indicate that in normoxic conditions, elevated NHE1 expression does not alter contractile function. During metabolic recovery, however, elevated NHE1 expression increased diastolic Ca^{2+} loading that led to augmented cell contractility.

Key words: heart failure, Na^+/H^+ exchanger, metabolic inhibition, metabolic recovery, contractile function, Ca^{2+} transient.

Résumé : Dans le myocarde, l'isoforme 1 de l échangeur Na^+/H^+ (NHE1) joue un rôle central dans la médiation de la lésion d'ischémie–reperfusion (I/R) en provoquant l'accumulation de Na^+ intracellulaire qui entraîne une surcharge de Ca^{2+}. L'un des principaux corrélats cliniques de la lésion d'I/R est la dysfonction contractile, dans laquelle la surcharge de Ca^{2+} causée par l'augmentation de l'échange Na^+/Ca^{2+} est un acteur important. Pour mieux comprendre le rôle cellulaire de NHE1 durant la lésion d'I/R, on a mesuré la fonction contractile et les augmentations transitoires de calcium durant l'inhibition et le rétablissement métabolique dans des myocytes ventriculaires unitaires de souris transgéniques présentant une forte expression de NHE1. En condition normoxique, aucune différence n'a été observée dans le raccourcissement cellulaire fractionnaire (RFC), le taux de raccourcissement (+dL/dt) et le taux de relaxation (-dL/dt) dans les cardiomyocytes surexprimant NHE1 versus type sauvage (TS). Lorsque le rétablissement métabolique a suivi l'inhibition métabolique, les myocytes ventriculaires surexprimant NHE1 on présenté une augmentation significative du RCF (130,2 % ± 11,77 % base) et des ±dL/dt (146,93 % ± 12,27 % base). Cette augmentation a concordé avec une augmentation significative de la concentration diastolique en calcium intracellulaire qui a été atténuée par l'inhibiteur de NHE1 HOE694. Ces résultats indiquent qu'en condition normoxique, une forte expression de NHE1 ne modifie pas la fonction contractile. Toutefois, durant le rétablissement métabolique, l'augmentation de l'expression de NHE1 a augmenté la charge diastolique en Ca^{2+}, ce qui a entraîné une augmentation de la contractilité cellulaire.

Mots-clés : insuffisance cardiaque, échangeur Na^+/H^+, inhibition métabolique, rétablissement métabolique, fonction contractile, augmentation transitoire du Ca^{2+}.

[Traduit par la Rédaction]

Introduction

Acute coronary occlusion is the leading cause of morbidity and mortality in the developed world. Although restoration of blood flow is an absolute prerequisite for the survival of the ischemic myocardium, reperfusion itself may lead to accelerated and additional myocardial injury, including hypercontracture and arrhythmias (Moens et al. 2005). The pathogenesis of this ischemia–reperfusion (I/R) injury involves a number of mechanisms. Increasing evidence suggests that elevated intracellular calcium (Ca^{2+} overload) plays an important role in the development of I/R injury

Received 8 July 2008. Accepted 2 October 2008. Published on the NRC Research Press Web site at cjpp.nrc.ca on 2 December 2008.

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 doi:10.1139/Y08-092 © 2008 NRC Canada

and that an important contributor to this increased intracellular calcium is the reverse mode of the Na^+/Ca^{2+} exchanger (Li et al. 1989; Moens et al. 2005).

Elevation of intracellular calcium and reversal of the Na^+/Ca^{2+} exchanger during I/R injury may result from activation of the Na^+/H^+ exchanger (NHE). NHE is a plasma membrane glycoprotein that extrudes one intracellular proton in exchange for one extracellular sodium, thus playing a crucial role in pH and volume regulation of the cell (Fliegel 2005). Of the 9 isoforms identified, NHE isoform 1 (NHE1) is the main isoform in cardiac tissue (Karmazyn et al. 2005). NHE1 has been implicated in various cardiac pathologies, including myocardial I/R injury (Karmazyn et al. 2005) and cardiac hypertrophy (Karmazyn 2001). During myocardial I/R, the decrease in intracellular pH leads to increased NHE1 activity, resulting in an elevated level of intracellular sodium. This promotes the reverse mode of the Na^+/Ca^{2+} exchanger, leading to Ca^{2+} overload and the subsequent development of contractile dysfunction, resulting in hypercontracture and arrhythmias (Avkiran 2001; Karmazyn et al. 2001; Lazdunski et al. 1985). Inhibition of NHE1 prevents I/R injury in various animal models (Ayoub et al. 2003; Cox et al. 2002; Knight et al. 2001), and genetic ablation of NHE1 has been shown to protect the heart against I/R injury (Wang et al. 2003), providing further evidence for this mechanism.

NHE expression levels can vary widely in different tissues, including the heart. Stimuli such as the chronic acidosis that accompanies ischemia increase protein and mRNA levels in the heart and other tissues (Dyck et al. 1995; Gan et al. 1999; Krapf et al. 1991). It is not clear, however, how increased levels and activity of NHE1 influence calcium homeostasis and contractile function in the myocardium. We previously showed that elevated levels of the NHE1 protein were not detrimental in isolated perfused hearts subjected to global ischemia (Imahashi et al. 2007). In the present study, we investigated the effects of NHE1 overexpression and activity using single ventricular cardiomyocytes isolated from transgenic mice overexpressing NHE1 in the myocardium. This allowed us to examine calcium fluxes on a beat-to-beat basis in individual cells. Single ventricular cardiomyocytes were subjected to metabolic inhibition followed by a 'recovery' period equivalent to the reoxygenation period seen in in vivo models. Our study provides novel evidence of NHE1 involvement in metabolic ischemia, and we demonstrate for the first time how elevated levels of NHE1 affect Ca^{2+} homeostasis in the myocardium during the contraction–relaxation cycle.

Materials and methods

Animal care and construction of transgenic mice with elevated NHE1 expression

Transgenic mice that specifically expressed additional wild-type NHE1 in the mouse myocardium were used in this study, as described earlier (the N-line transgenic mouse) (Imahashi et al. 2007). Briefly, the hemagglutinin (HA)-tagged human NHE1 was obtained from the plasmid pYN4+ (Slepkov et al. 2005) and was directed to the myocardium by using the mouse α-myosin heavy chain (MHC) promoter. All animals were used in accordance with guidelines set out by the Canadian Council on Animal Care.

NHE1 protein expression

To confirm that NHE1 was specifically expressed in the myocardium, lysates were prepared from heart, liver, kidney, lungs, and brain tissue (Imahashi et al. 2007). The membrane fraction containing the pellet was resuspended in buffer containing 1% SDS for solubilization. Protein concentration was quantified by using the Bio-Rad DC protein assay kit. Western blot immunoreactivity was quantified after incubation with either the rabbit polyclonal anti-HA tag (Santa Cruz sc805 Y-11) or mouse monoclonal anti-NHE1 (BD Pharmingen, USA). Actin was used as a loading control. Quantification was with the LI-COR Biosciences Odyssey infrared imaging system. To generate a positive control, AP-1 cells, a derivative of Chinese hamster ovary cells that lacks endogenous NHE activity, were transfected with the HA-tagged human NHE1 protein obtained from the pYN4+ plasmid (Slepkov et al. 2005). Lysates of AP-1 cells were made as described earlier (Murtazina et al. 2001).

Isolation of ventricular myocytes

Seven- to eight-week-old NHE1-transgenic (+NHE1) mice or wild-type (WT) littermates were anesthetized with halothane and euthanized with cervical dislocation. The hearts were removed and ventricular myocytes were isolated by enzymatic dissociation as described earlier (Baczko et al. 2005; Imahashi et al. 2007). After isolation, cells were maintained in storage buffer at 25 °C for a minimum of 2 h before use.

Ca^{2+} transients and cell-shortening measurement of isolated ventricular myocytes

Freshly isolated ventricular myocytes were examined by using the calcium-sensitive fluorescent probe Calcium Green-1 AM as described earlier (Baczko et al. 2005). Cells were field-stimulated at 1 Hz with 2-millisecond square pulses at a constant current 20% above threshold; a photomultiplier detection system was used for data acquisition and analysis (Baczko et al. 2005). The increase in fluorescence intensity was normalized to the amplitude of the Ca^{2+} transient recorded under baseline conditions in each experiment. Cell shortening was measured by using a video edge detection system (Crescent Electronics, Salt Lake City, USA) at 60 Hz frame rate. Cell shortening was expressed as fractional shortening ($\Delta L/L_0$, where L_0 is the resting cell length). Ventricular myocytes were subjected to a metabolic inhibition protocol. After superfusion with control solution, cells were superfused with control solution containing 4 mmol/L NaCN and 5 mmol/L 2-deoxyglucose for 6 min of metabolic inhibition followed by 8 min of recovery with control solution in the presence or absence of the NHE1 inhibitor HOE694 (5 μmol/L). The return to control solution in the absence of metabolic inhibitors was referred to as 'metabolic recovery.' Cell shortening and Ca^{2+} transient measurements were carried out at room temperature (22 ± 1 °C).

Statistical analysis

Data were expressed as means ± SE. Significance was determined by Student's paired or unpaired t test or ANOVA, as appropriate; $p < 0.05$ was considered statistically significant.

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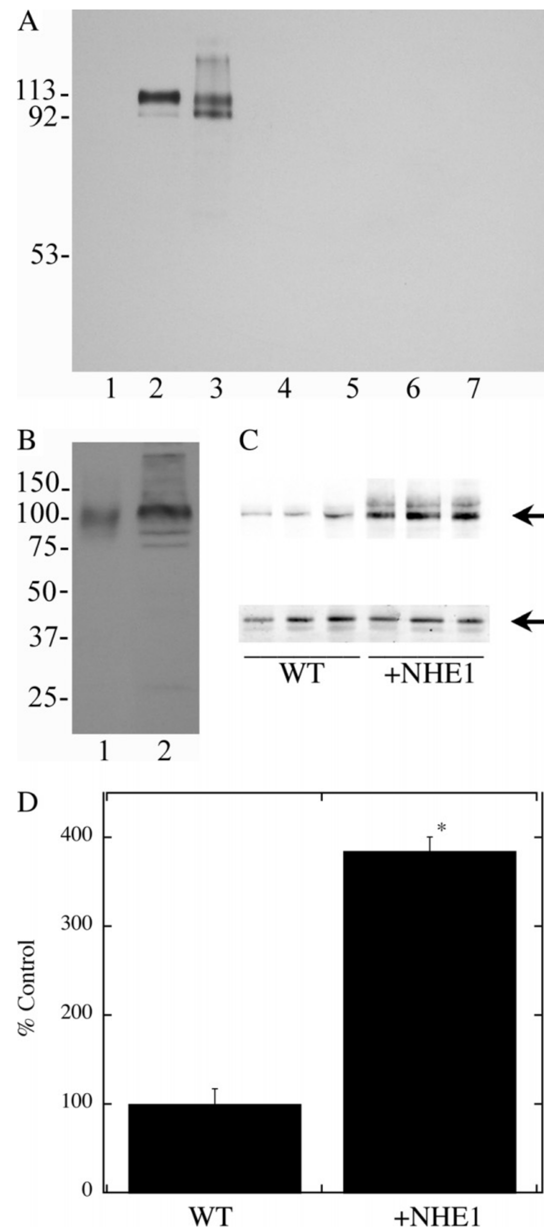

Fig. 1. Western blot analysis of transgenic mice examined for expression of NHE1. (A) Tissue-specific expression of NHE1-transgenic mice. Lane 1 is an extract of a wild-type heart. Lane 2 is a positive control, a cell extract from AP-1 cells transfected with HA-tagged NHE. Lanes 3–7 are extracts from the heart, lung, liver, kidney and brain, respectively, from mice with the NHE1 transgene. (B) Western blot against isolated cardiomyocytes for expression of the HA-tagged NHE. Lane 1, positive control of cell extract from AP-1 cells transfected with HA-tagged NHE. Lane 2, cell extract from freshly isolated cardiomyocytes from NHE1-transgenic mice. (C) Western blot of heart cell extracts from control and +NHE1 hearts. Arrow denotes location of immunoreactive species. Upper panel, NHE1 immunoblot; lower panel, probing with anti-actin antibodies. (D) Summary of NHE1 expression levels (relative to actin) in WT and +NHE1 mouse heart cell extracts. Values are means ± SE, $n = 4$. Asterisk (*) indicates significant difference from controls ($p < 0.05$). NHE1, Na^+/H^+ exchanger isoform 1; WT, wild-type; AP-1, a cell line that lacks endogenous NHE activity; HA, hemagglutinin; +NHE1, NHE1-transgenic mice.

Results

Characterization of transgenic mice with elevated NHE1 expression

We examined both exogenous and total NHE1 protein in the transgenic mice to confirm that the levels of the NHE1 protein were elevated and specifically targeted to the myocardium. Figure 1A demonstrates the cardiac-specific overexpression of the NHE1 transgene in +NHE1 mice. Lane 1 is an extract from WT mouse heart that shows no immunoreactivity to anti-HA. Lane 2 is an extract of a positive control made from a stable NHE-deficient AP-1 cell line transfected with HA-tagged NHE1. The predominant immunoreactive species is 110 kDa; a secondary band of size approximately 90–95 kDa was also found. The second immunoreactive band has previously been suggested to represent unglycosylated or partially glycosylated protein (Slepkov et al. 2005). Lane 3 demonstrates the immunoreactivity that was present in the +NHE1 mouse myocardium. There was no detectable expression in the lung, liver, kidney, or brain (Lanes 4–7, respectively). To confirm that exogenous NHE1 was expressed in isolated cardiomyocyte cells per se, freshly isolated cardiomyocytes from +NHE1 mice were used for Western blotting with the anti-HA tag (Fig. 1B). Lane 1 is a positive control generated from AP-1 cells transfected with HA-tagged NHE1. Lane 2 is a sample of cardiomyocytes, freshly isolated from +NHE1 mice. The HA-tagged NHE1 protein is visible at 110 kDa in the cardiomyocyte sample, demonstrating for the first time that the exogenous NHE1 protein is present in this particular cell type. To examine the levels of total exogenous and endogenous NHE1 protein, we used an antibody against the NHE1 protein (and not the HA tag) (Figs. 1C, 1D). Figure 1C shows a Western blot with anti-NHE1 antibodies (upper panel) and anti-actin antibodies (lower panel). NHE1 expression was greatly elevated in the transgenic mice. Figure 1D shows quantification of the immunoreactivity and demonstrates for the first time that total NHE1 protein expression is approximately 3-fold greater in +NHE1 than in WT (Fig. 1D). Previously we demonstrated that NHE activity of isolated +NHE1 ventricular myocytes was approximately 3-fold greater than that of isolated WT ventricular myocytes (Imahashi et al. 2007), which is similar to the present results for protein levels.

Effects of elevated NHE1 expression on contractile function during normoxic conditions

Detailed analysis of cell shortening of ventricular myocytes isolated from WT and +NHE1 animals showed no difference in fractional cell shortening (FCS), nor in contractility and relaxation as measured by maximal rate of cell shortening (+dL/dt) and maximal rate of relengthening (–dL/dt) during 1 Hz field stimulation (Figs. 2A–2D). These results indicate that, by itself, elevated cardiac-specific expression of NHE1 does not lead to contractile dysfunction under normoxic (baseline) conditions.

Because we used the NHE1 inhibitor HOE694 in the latter part of this study, a set of experiments was performed to test the effect of HOE694 on cell shortening both in WT and +NHE1 myocytes at baseline, under normoxic condi-

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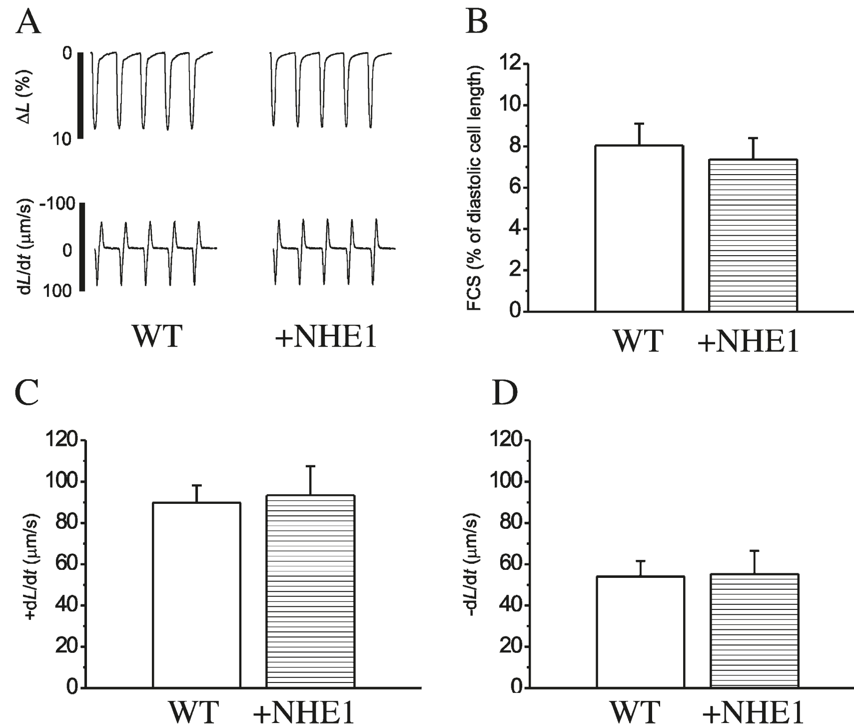
Fig. 2. Effects of elevated cardiac-specific expression of the NHE1 protein on contractile function of single ventricular cardiomyocytes during normoxic conditions. (A) Representative recordings of cell shortening from wild-type (WT) and NHE1-transgenic mice. (B) Grouped data of fractional cell shortening of WT and +NHE1 mice. (C) maximal rate of shortening and (D) relengthening of WT and +NHE1 mice. n = 15–19 cells/group. NHE1, Na^+/H^+ exchanger isoform 1; WT, wild-type; +NHE1, NHE1-transgenic mice; FCS, fractional cell shortening; +dL/dt, rate of shortening; –dL/dt, rate of relaxation.



tions. These experiments revealed that 5 μmol/L HOE694 had no effect on FCS, +dL/dt, or –dL/dt (data not shown).

Effects of elevated NHE1 expression on contractile function during metabolic inhibition and recovery

To test the effect of elevated NHE1 expression on contractile function during metabolic recovery, cells were subjected to 6 min of metabolic inhibition followed by 8 min of recovery with control solution in the presence or absence of 5 μmol/L HOE694. Figures 3A and 3B examine the effect of metabolic inhibition on WT and +NHE1 cardiomyocytes. +NHE1 ventricular myocytes exhibited increased FCS and increased ±dL/dt values at the end of the metabolic inhibition period compared with those at baseline. The increase in FCS was significantly higher in +NHE1 myocytes. For WT cardiomyocytes, FCS in normoxic conditions was 7.88 ± 0.86% of basal diastolic cell length, and after metabolic inhibition it was 8.67 ± 1.66% (or 112.5% of baseline). For +NHE1 cardiomyocytes, FCS at baseline was 7.37 ± 1.04%, and after metabolic inhibition it was 11.31 ± 1.43%. This change in FCS represented an increase of 153.5% of baseline in +NHE1 cardiomyocytes.

After metabolic inhibition, there were significant differences between WT and +NHE1 cardiomyocytes during metabolic recovery (Figs. 3C–3D). The metabolic recovery period in WT cardiomyocytes resulted in a significant decrease in FCS, relaxation (–dL/dt), and shortening (+dL/dt), and relaxation was affected more than shortening (FCS: 81.5% ± 9.31% baseline; –dL/dt: 48.4% ± 10.45% baseline; +dL/dt: 72.3% ± 10.52% baseline) (Figs. 3C–3E). On the other hand, +NHE1 ventricular myocytes showed a significant increase in FCS (130.2% ± 11.77% baseline) and contractility (146.93% ± 12.27% baseline). Relaxation rates of +NHE1 ventricular myocytes were similar to those in baseline conditions (113.1% ± 12.01% baseline), but were elevated in comparison with WT cardiomyocytes (Fig. 3F). The NHE1 inhibitor HOE694 improved FCS, contractility, and relaxation in WT myocytes, but did not further improve these parameters in +NHE1 ventricular myocytes (Figs. 3D–3F).

Effects of elevated NHE1 expression on Ca^{2+} transients during metabolic recovery

To determine how elevated NHE1 levels affected contractility, we examined calcium transients of the ventricular myocytes subjected to metabolic inhibition followed by metabolic recovery. The observed changes in contractile function of +NHE1 ventricular myocytes were paralleled by a significant increase in diastolic intracellular calcium during metabolic recovery. Diastolic intracellular calcium was approximately 2-fold greater than that of control ventricular myocytes (Figs. 4A, 4C, 4E). HOE694 significantly decreased metabolic recovery-induced diastolic Ca^{2+} loading in +NHE1 ventricular myocytes (Figs. 4C–4E), although the level of diastolic intracellular calcium was still significantly elevated in comparison with that of WT.

Discussion

I/R injury causes hypercontracture, electrical dysfunction, and death of injured myocytes (van Borren et al. 2004). The

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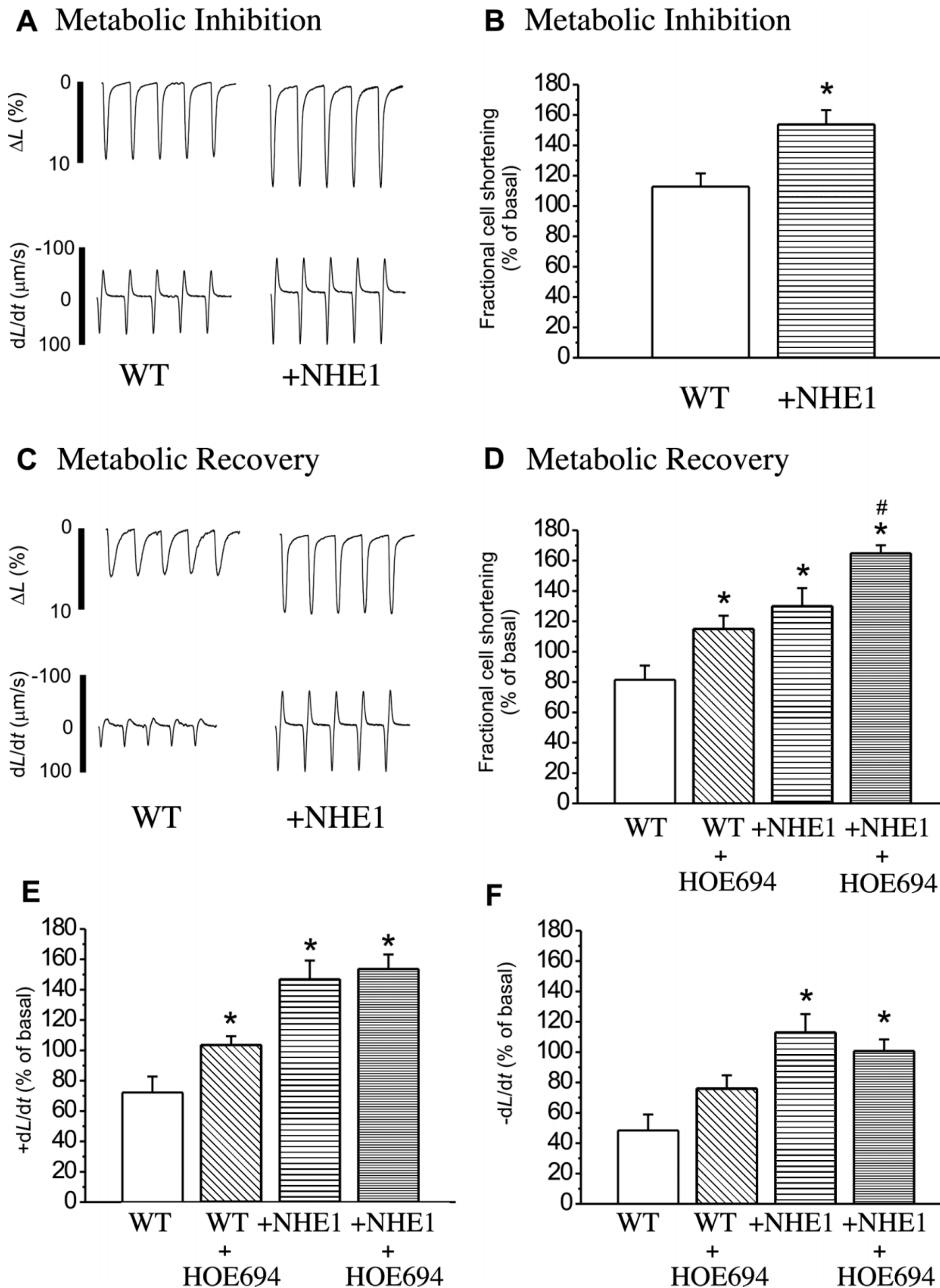
Fig. 3. Effects of metabolic inhibition, and metabolic inhibition followed by metabolic recovery, on contractile parameters of wild-type and NHE1-transgenic mouse cardiomyocytes. Metabolic inhibition was applied for 6 min and was followed by 8 min recovery with and without NHE1 inhibitor HOE694 (5 μmol/L). (A, B) Representative cell-shortening recordings (A) and grouped data (B) showing the effect of metabolic inhibition on isolated cardiomyocytes from WT and +NHE1 mice. (C–F) Effect of metabolic inhibition followed by metabolic recovery on contractile parameters of WT and +NHE1 mouse cardiomyocytes. Representative tracings (C) and summary of data (D) for fractional cell shortening. (D) Effect of HOE694 on FCS in WT and +NHE1 cardiomyocytes. (E) Contractility depicted by the maximal rate of shortening of WT and +NHE1 cardiomyocytes in the presence or absence of HOE694 during reoxygenation. (F) Maximal rate of relengthening of ventricular cardiomyocytes isolated from WT and +NHE1 mice treated with HOE694 during reoxygenation. The concentration of the NHE1 inhibitor HOE694 was 5 μmol/L in all experiments. n = 5–7 cells per group. *, Significant at $p < 0.05$ vs. WT. #, $p < 0.05$ vs. NHE1 group. WT, wild-type; NHE1, Na^+/H^+ exchanger isoform 1; +NHE1, NHE1-transgenic mice; +dL/dt, rate of shortening; –dL/dt, rate of relaxation.



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pathophysiologic chain of events revolves around Ca^{2+} overload resulting from energy deprivation and the consequent liberation of protons. The latter is thought to stimulate NHE1, which has been implicated in playing a key role in myocardial I/R injury (Karmazyn et al. 1999). Despite numerous studies showing that inhibition of NHE1 is cardioprotective, the effects of elevated NHE1 expression and activity on contractile function and ionic homeostasis have not been investigated. This is of particular interest because several studies have demonstrated that an elevation in NHE1 expression occurs in various pathologic settings in the myocardium (Dyck et al. 1995; Gan et al. 1999), including human hearts with chronic end-stage heart failure (Yokoyama et al. 2000). In this study, we investigated the pathophysiologic role of increased NHE1 expression in the myocardium using transgenic mice overexpressing NHE1 specifically in the myocardium. We confirmed in the present study, and previously (Imahashi et al. 2007), that NHE1 is specifically targeted to the myocardium and that NHE1 activity increases by approximately 3-fold when compared with that of controls. In addition, we previously demonstrated that expression of several ionic homeostatic proteins (Na^+/K^+-ATPase, Na^+/Ca^{2+} exchanger, sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA), sodium bicarbonate cotransporter, and anion exchanger type 3 isoform) did not change in the myocardium of +NHE1 mice (Imahashi et al. 2007). This suggests that any changes we observed are most likely initiated as a result of changes in NHE1 activity.

Our initial comparisons of WT and +NHE1 isolated ventricular cardiomyocytes were at baseline normoxic conditions. In this case we did not detect any difference in contractile function. Similar results were obtained with in vivo NHE1-overexpressing hearts, in which no changes were detected at baseline in heart performance, resting intracellular pH, or phosphocreatine/ATP levels (Imahashi et al. 2007). This data suggest that increased cardiac-specific expression of the normal NHE1 does not cause any gross alteration of cardiac function under normal circumstances.

Multiple interdependent processes play a role in the pathologic phenomena associated with myocardial I/R injury, but Ca^{2+} overload appears to be a major component (Eisner et al. 1989; Hayashi et al. 1992; Li et al. 1989). For this reason we examined Ca^{2+} levels in cardiomyocytes with elevated NHE1 activity. Because NHE1 plays a key role in Na^+ and Ca^{2+} loading during I/R, we examined the effects of elevated NHE1 expression in a cellular model of metabolic inhibition and recovery. This allowed us to monitor intracellular calcium on a beat-to-beat basis. We have previously found this model of metabolic inhibition and recovery to be an excellent model that mimics events that occur during I/R injury (Baczko et al. 2003, 2005). Although not a true model of I/R, it allows us to study reversible injury with close parallels to ischemia but during a much shorter time period that is controllable and reproducible. Others have previously used this and similar models in cardiomyocytes to study I/R or metabolic injury and calcium fluxes (Eisner et al. 1989; Hayashi et al. 1992; Li et al. 1989).

When isolated cardiomyocytes were subjected to metabolic inhibition and recovery, cells expressing elevated NHE1 exhibited significantly increased FCS and increased $\pm dL/dt$. Surprisingly, the NHE1 inhibitor HOE694 increased the effects on FCS but did not significantly change effects on $\pm dL/dt$. These results were suggestive of an acceleration of calcium entry through the pathway involving NHE1 and reverse-mode Na^+/Ca^{2+} exchanger activity (Karmazyn 2003). To examine whether this course of events was involved, we examined calcium oscillations in the WT and +NHE1 cardiomyocytes. The results were most revealing and our major finding was that diastolic calcium was elevated in cardiomyocytes expressing elevated levels of NHE1. This was attenuated by treatment with HOE694, although calcium was still elevated relative to WT cardiomyocytes. Eisner et al. (1989) found similar but smaller and more variable effects in their study of the effects of metabolic ischemia on ventricular cells. Addition of cyanide alone had a variable effect, often causing increased contraction and increased calcium transients. Cyanide coupled with inhibition of glycolysis caused a decrease in the magnitude of calcium transients and a small increase in diastolic calcium. Our study used both cyanide and 2-deoxyglucose to inhibit glycolysis and oxidative phosphorylation. The elevation of diastolic calcium that we observed was much greater in +NHE1 cardiomyocytes. It should be noted, however, that we used isolated cardiomyocytes from mouse myocardium and Eisner et al. (1989) used rat ventricular cells that may not be completely comparable.

We noted an enhancement of contractility in +NHE1 cardiomyocytes after both metabolic inhibition and metabolic recovery. Our earlier study of isolated perfused hearts from these mice showed that, rather than the expected detrimental effects, recovery after global I/R was slightly improved with elevated expression of NHE1 in the myocardium (Imahashi et al. 2007). It is also worth note that a preliminary report from another laboratory (Cook et al. 2008) has also independently shown that elevated NHE1 expression in transgenic mice reduced susceptibility to ischemia reperfusion injury and enhanced recovery. Therefore, the results in the present study are consistent with our earlier findings, and those of others, in intact hearts. However, the mechanism by which enhanced NHE1 levels are beneficial in our system remains elusive. This is further complicated by the observation that treatment of cells with HOE694 did not eliminate this effect. Our observation that elevated NHE1 activity enhanced diastolic calcium levels provides some insight to these results, as it suggests that NHE1 may mediate its effects through reversal of the Na^+/Ca^{2+} exchanger. This suggestion is supported by another study that showed that diastolic calcium elevation is caused by the reverse mode of Na^+/Ca^{2+}-exchanger activity (Wei et al. 2007). Figure 5 summarizes a possible mechanism by which enhanced NHE activity may increase diastolic calcium. The mechanism is similar to that reported earlier for detrimental effects of NHE1 on the myocardium (Karmazyn et al. 2001), but may vary in degree. Metabolic inhibition results in decreased intracellular ATP production through decreased aerobic metabolism. This results in increased intracellular protons as a product of metabolism and elevated intracellular sodium from both decreased Na^+/K^+-ATPase activity and increased NHE1 activity. The increased intracellular sodium results in a reversal of Na^+/Ca^{2+}-exchanger activity and increased intracellular calcium, which would be taken up into intracellular calcium stores and contribute in the cycle of excitation

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Fig. 4. Calcium transient recordings from wild-type and NHE1-overexpressing isolated cardiomyocytes during metabolic recovery with and without NHE1 inhibitor HOE694 (5 µmol/L). (A–D) Representative recordings of complete experiments with expanded regions as noted by arrows. (E) Grouped data expressed as a percentage increase in diastolic Ca^{2+} indicator fluorescence. Dotted line illustrates control diastolic Ca^{2+} indicator fluorescence level. n = 5–9 cells per group. *, Significant at $p < 0.05$ vs. Wt. #, $p < 0.05$ vs. NHE1 group. WT, wild-type; NHE1, Na^+/H^+ exchanger isoform 1; +NHE1, NHE1-transgenic mice; MI, myocardial infarction.

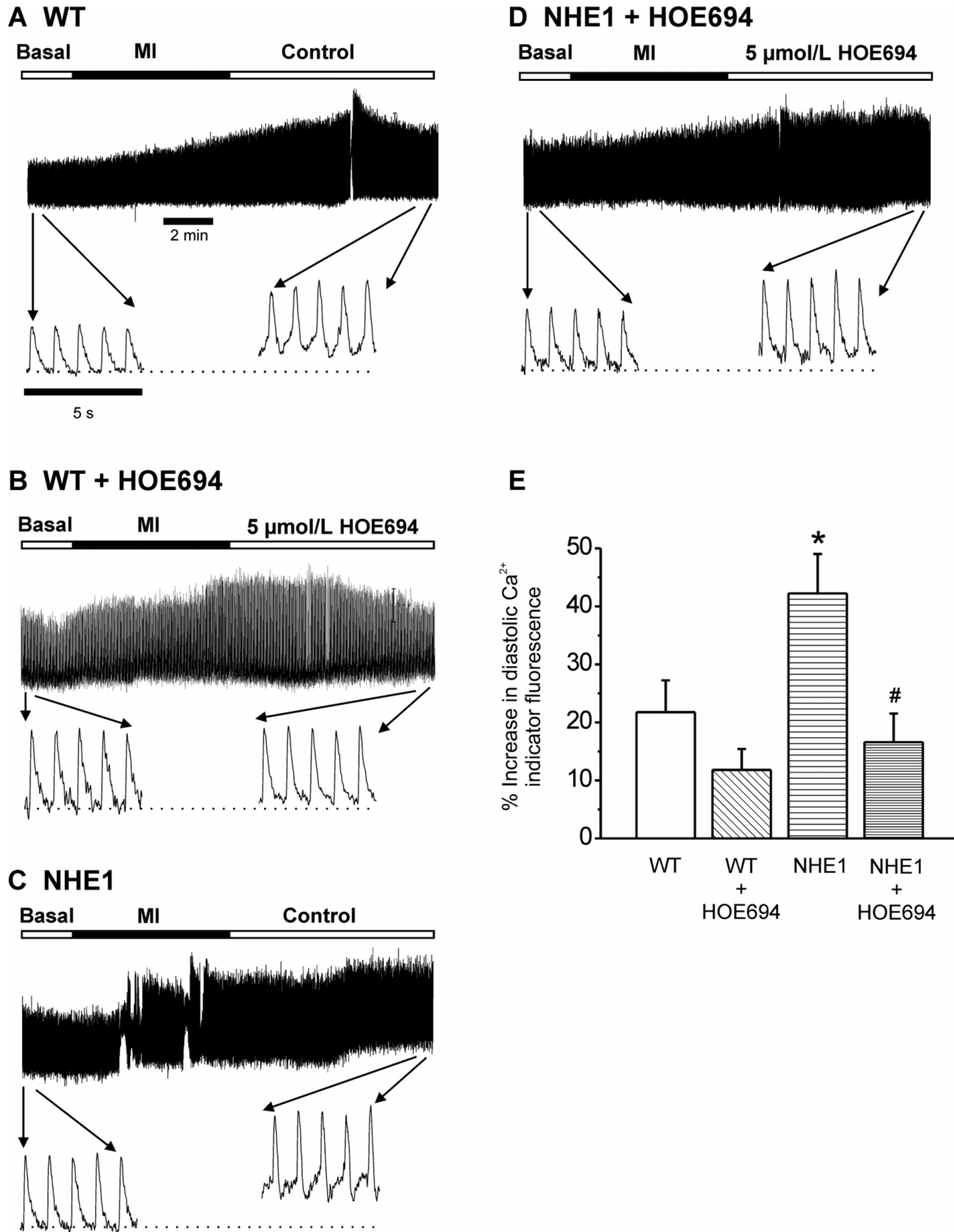

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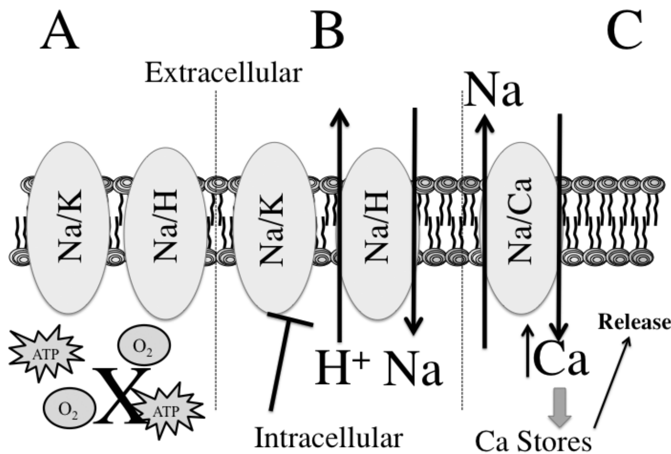
Fig. 5. Schematic diagram of the mechanism by which metabolic ischemia may affect intracellular calcium levels in isolated cardiomyocytes. (A) Metabolic ischemia causes decreased intracellular oxygen with resultant decreases in ATP production. (B) Decreased intracellular ATP reduces the activity of the plasma membrane Na^+/K^+-ATPase. In addition, there is increased intracellular H^+ production from anaerobic metabolism. Decreased sodium extrusion and increased activity of the Na^+/H^+ exchanger results in increased intracellular sodium. (C) Increased intracellular sodium results in reversal of activity of the Na^+/Ca^{2+} exchanger and increased intracellular calcium.



and contraction. Decreased activity of Na^+/K^+-ATPase and increased NHE1 activity have been demonstrated previously as a result of metabolic inhibition, and increases in intracellular calcium through the Na^+/Ca^{2+} exchanger have been suggested to be the result (Hayashi 2000). Elevating calcium entrance through the plasma membrane would result in uptake and release of calcium from intracellular stores during the excitation–contraction cycle. Thus metabolic ischemia, together with elevated NHE1 expression, may serve as an agent that facilitates intracellular calcium mobilization and possibly increases myofibrillar sensitivity to calcium (Endoh 1998), with resulting enhancement of contractility.

HOE694 reduced much of the elevation of diastolic calcium in +NHE1 cardiomyocytes, although it was still significantly elevated compared with that of WT cardiomyocytes. It is possible that NHE1 inhibition was not complete, resulting in the partial elevation of calcium levels though the mechanism described above (Fig. 5). Alternatively, sodium from other sources might lead to elevated calcium. This might include inhibition of the Na^+/K^+-ATPase through depletion of intracellular ATP levels. In addition, there was certainly still beat-to-beat opening of voltage-gated sodium channels during the action potential that could contribute to elevation in intracellular sodium.

We noted that in the calcium transient recordings (Fig. 4A) there appeared to be an occasional afterdepolarization-induced calcium fluctuation in the +NHE1 cardiomyocytes. Although this was not a common observation, it occurred several times in the +NHE1 cardiomyocytes and not in WT. This may reflect the effects of elevated diastolic calcium, similar to arrythmogenesis, that is reported to be increased by elevated intracellular calcium through the Na^+/Ca^{2+} exchanger (Tanaka et al. 2007). Elevated NHE1 expression may therefore be proarrhythmic as a result of elevated calcium levels. This supports the observation of previous studies that showed that NHE1 inhibition attenuated reperfusion-induced arrhythmias in the rat myocardium (Ono et al. 2004). Further experiments are necessary to confirm that elevated NHE1 levels promote arrhythmias.

It may be that the enhancement of contractility we observed in +NHE1 cardiomyocytes is due, in part, to a positive inotropic-like effect. Ouabain is well known to cause positive inotropic effects by inhibition of Na^+/K^+-ATPase activity, subsequent elevation of intracellular Na^+, and elevation of intracellular calcium through reverse mode activity of the Na^+/Ca^{2+} exchanger (Tanaka et al. 2007). Given that metabolic inhibition and NHE1 activity also act through a similar mechanism (Fig. 5), contractility may be enhanced through this mechanism. This enhanced contractility may be mitigated by inhibition of NHE1 activity, which reduced, but did not eliminate, elevated diastolic calcium. An alternative explanation is that the enhancement of contractility we observed in +NHE1 cardiomyocytes was due to increased rates of proton extrusion. Intracellular acidosis has detrimental effects on cardiac contractility (Jeffrey et al. 1987; Katz and Hecht 1969).

Numerous studies have shown that inhibition of NHE1 improves recovery after I/R injury (Ayoub et al. 2003; Cox et al. 2002; Knight et al. 2001). Therefore it would seem that elevated NHE1 expression should result in further detrimental effects, yet these were not seen in this and our earlier study (Imahashi et al. 2007). Why did this occur? Isolated cardiomyocytes do not have the workload of the intact myocardium; therefore they may not exacerbate the detrimental effects of increased intracellular Ca^{2+}. The present experimental model may not have been severe enough to cause damage to the cardiomyocytes. Alternatively, there may be some as yet undetermined beneficial advantage to having elevated NHE1 expression. It is presently unknown what this could be, but there are some suggestions that this is the case. First, in this study, in our previous study (Imahashi et al. 2007), and in a preliminary report by others (Cook et al. 2008), elevated NHE1 expression was beneficial and not detrimental. Second, it is clear that elevated NHE1 expression has evolved to occur in response to several disease states in the heart and other tissues (Dyck et al. 1995; Gan et al. 1999; Krapf et al. 1991), and it appears unlikely that this would occur to the detriment of the myocardium. Cook et al. (2008) suggested that increased NHE1 expression may induce a protective ER stress response that could enhance cardiac contractility, and this could account for resistance to ischemic reperfusion injury. From the standpoint of evolutionary adaptation, although it is true that complete, or near-complete, pharmacologic inhibition of NHE1 is beneficial to the myocardium, this may be impossible to reproduce in vivo physiologically for mammalian cells. In the heart, a more beneficial approach in the short term may be to upregulate levels of the protein. NHE1 is also known to play important roles in cell motility, in anchoring the cytoskeleton, and in apoptosis (Malo and Fliegel 2006). It is also possible that in one of these other functions of NHE1, its upregulation is important in response to stress in the myocardium. Further studies are needed to investigate this phenomenon.

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Acknowledgements

This work was supported by grants to L.F. and P.E.L. from the Canadian Institutes for Health Research (CIHR). L.F. is an Alberta Heritage Foundation for Medical Research Senior Scientist. P.E.L. is an Alberta Heritage Foundation for Medical Research Senior Scholar. I.B. and F.M. were partially supported by the CIHR Strategic Training Initiative in Membrane Proteins and Cardiovascular Disease. F.M. was also supported by a CIHR doctoral training award.

References

Avkiran, M. 2001. Protection of the ischaemic myocardium by Na^+/H^+ exchange inhibitors: potential mechanisms of action. Basic Res. Cardiol. **96**: 306–311. doi:10.1007/s003950170037. PMID:11518185.

Ayoub, I.M., Kolarova, J., Yi, Z., Trevedi, A., Deshmukh, H., Lubell, D.L., et al. 2003. Sodium-hydrogen exchange inhibition during ventricular fibrillation: beneficial effects on ischemic contracture, action potential duration, reperfusion arrhythmias, myocardial function, and resuscitability. Circulation, **107**(13): 1804–1809. doi:10.1161/01.CIR.0000058704.45646.0D. PMID:12665493.

Baczko, I., Giles, W.R., and Light, P.E. 2003. Resting membrane potential regulates Na(+)-Ca^{2+} exchange-mediated Ca^{2+} overload during hypoxia-reoxygenation in rat ventricular myocytes. J. Physiol. **550**(Pt 3): 889–898. doi:10.1113/jphysiol.2003.043372. PMID:12807988.

Baczko, I., Jones, L., McGuigan, C.F., Manning Fox, J.E., Gandhi, M., Giles, W.R., et al. 2005. Plasma membrane K_{ATP} channel-mediated cardioprotection involves posthypoxic reductions in calcium overload and contractile dysfunction: mechanistic insights into cardioplegia. FASEB J. **19**(8): 980–982. doi:10.1096/fj.04-3008fje. PMID:15774423.

Cook, A.R., Bardswell, S.C., Pretheshan, S., Dighe, K., Kanaganayagam, G.S., Jabr, R.I., et al. 2008. Paradoxical resistance to myocardial ischemia and age-related cardiomyopathy in NHE1 transgenic mice: a role for ER stress? J. Mol. Cell. Cardiol. doi:10.1016/j.yjmcc.2008.10.013. In press.

Cox, C.S., Jr., Sauer, H., Allen, S.J., Buja, L.M., and Laine, G.A. 2002. Sodium/hydrogen-exchanger inhibition during cardioplegic arrest and cardiopulmonary bypass: an experimental study. J. Thorac. Cardiovasc. Surg. **123**(5): 959–966. doi:10.1067/mtc.2002.120715. PMID:12019382.

Dyck, J.R.B., Maddaford, T., Pierce, G.N., and Fliegel, L. 1995. Induction of expression of the sodium-hydrogen exchanger in rat myocardium. Cardiovasc. Res. **29**: 203–208. PMID:7736496.

Eisner, D.A., Nichols, C.G., O'Neill, S.C., Smith, G.L., and Valdeolmillos, M. 1989. The effects of metabolic inhibition on intracellular calcium and pH in isolated rat ventricular cells. J. Physiol. **411**: 393–418. PMID:2614727.

Endoh, M. 1998. Changes in intracellular Ca^{2+} mobilization and Ca^{2+} sensitization as mechanisms of action of physiological interventions and inotropic agents in intact myocardial cells. Jpn. Heart J. **39**(1): 1–44. PMID:9601480.

Fliegel, L. 2005. The Na^+/H^+ exchanger isoform 1. Int. J. Biochem. Cell Biol. **37**(1): 33–37. doi:10.1016/j.biocel.2004.02.006. PMID:15381146.

Gan, X.T., Chakrabarti, S., and Karmazyn, M. 1999. Modulation of Na^+/H^+ exchange isoform 1 mRNA expression in isolated rat hearts. Am. J. Physiol. **277**: H993–H998. PMID:10484421.

Hayashi, H. 2000. Pathogenesis and the role of Ca2+ overload during myocardial ischemia/reperfusion. Nagoya J. Med. Sci. **63**(3–4): 91–98. PMID:11201989.

Hayashi, H., Miyata, H., Noda, N., Kobayashi, A., Hirano, M., Kawai, T., and Yamazaki, N. 1992. Intracellular Ca^{2+} concentration and pH_i during metabolic inhibition. Am. J. Physiol. **262**(3 Pt 1): C628–C634. PMID:1550207.

Imahashi, K., Mraiche, F., Steenbergen, C., Murphy, E., and Fliegel, L. 2007. Overexpression of the Na^+/H^+ exchanger and ischemia-reperfusion injury in the myocardium. Am. J. Physiol. Heart Circ. Physiol. **292**(5): H2237–H2247. doi:10.1152/ajpheart.00855.2006. PMID:17209001.

Jeffrey, F.M.H., Mallow, C.R., and Radda, G.K. 1987. Influence of intracellular acidosis on contractile function in the working rat heart. Am. J. Physiol. **253**: H1499–H1505. PMID:3425750.

Karmazyn, M. 2001. Role of sodium-hydrogen exchange in cardiac hypertrophy and heart failure: a novel and promising therapeutic target. Basic Res. Cardiol. **96**(4): 325–328. doi:10.1007/s003950170040. PMID:11518188.

Karmazyn, M. 2003. Mechanisms underlying NHE-1 involvement in myocardial ischemic and reperfusion injury, pp. 221–237. *In* The Na^+/H^+ exchanger, from molecular to its role in disease. *Edited by* M. Karmazyn, M. Avkiran, and L. Fliegel. Kluwer Academic Publishers, Boston.

Karmazyn, M., Gan, T., Humphreys, R.A., Yoshida, H., and Kusumoto, K. 1999. The myocardial Na^+-H^+ exchange: structure, regulation, and its role in heart disease. Circ. Res. **85**: 777–786. PMID:10532945.

Karmazyn, M., Sostaric, J.V., and Gan, X.T. 2001. The myocardial Na^+/H^+ exchanger: a potential therapeutic target for the prevention of myocardial ischaemic and reperfusion injury and attenuation of postinfarction heart failure. Drugs, **61**(3): 375–389. doi:10.2165/00003495-200161030-00006. PMID:11293648.

Karmazyn, M., Sawyer, M., and Fliegel, L. 2005. The Na^+/H^+ exchanger: a target for cardiac therapeutic intervention. Curr. Drug Targets Cardiovasc. Haematol. Disord. **5**(4): 323–335. doi:10.2174/1568006054553417. PMID:16101565.

Katz, A.M., and Hecht, H.H. 1969. The early "pump" failure of the ischemic heart. Am. J. Med. **47**: 497–502. doi:10.1016/0002-9343(69)90180-6. PMID:4241836.

Knight, D.R., Smith, A.H., Flynn, D.M., MacAndrew, J.T., Ellery, S.S., Kong, J.X., et al. 2001. A novel sodium-hydrogen exchanger isoform-1 inhibitor, zoniporide, reduces ischemic myocardial injury in vitro and in vivo. J. Pharmacol. Exp. Ther. **297**: 254–259. PMID:11259552.

Krapf, R., Pearce, D., Lynch, C., Xi, X.P., Reudelhuber, T.L., Pouyssegur, J., and Rector, F.C.J. 1991. Expression of rat renal Na^+/H^+ antiporter mRNA levels in response to respiratory and metabolic acidosis. J. Clin. Invest. **87**(2): 747–751. doi:10.1172/JCI115057. PMID:1846882.

Lazdunski, M., Frelin, C., and Vigne, P. 1985. The sodium/hydrogen exchange system in cardiac cells: its biochemical and pharmacological properties and its role in regulating internal concentrations of sodium and internal pH. J. Mol. Cell. Cardiol. **17**: 1029–1042. doi:10.1016/S0022-2828(85)80119-X. PMID:3001319.

Li, Q., Hohl, C.M., Altschuld, R.A., and Stokes, B.T. 1989. Energy depletion-repletion and calcium transients in single cardiomyocytes. Am. J. Physiol. **257**(3 Pt 1): C427–C434. PMID:2782386.

Malo, M.E., and Fliegel, L. 2006. Physiological role and regulation of the Na^+/H^+ exchanger. Can. J. Physiol. Pharmacol. **84**(11): 1081–1095. doi:10.1139/Y06-065. PMID:17218973.

Moens, A.L., Claeys, M.J., Timmermans, J.P., and Vrints, C.J. 2005. Myocardial ischemia/reperfusion-injury, a clinical view on a complex pathophysiological process. Int. J. Cardiol. **100**(2): 179–190. doi:10.1016/j.ijcard.2004.04.013. PMID:15823623.

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Murtazina, R., Booth, B.J., Bullis, B.L., Singh, D.N., and Fliegel, L. 2001. Functional analysis of polar amino-acid residues in membrane associated regions of the NHE1 isoform of the mammalian Na^+/H^+ exchanger. Eur. J. Biochem. **268**(17): 4674–4685. doi:10.1046/j.1432-1327.2001.02391.x. PMID:11532004.

Ono, M., Nakamura, M., and Koga, Y. 2004. KB-R9032, newly developed Na^+/H^+ exchange inhibitor, attenuates reperfusion-induced arrhythmias in isolated perfused rat heart. J. Anesth. **18**(3): 196–202. doi:10.1007/s00540-004-0232-x. PMID:15290419.

Slepkov, E.R., Rainey, J.K., Li, X., Liu, Y., Cheng, F.J., Lindhout, D.A., et al. 2005. Structural and functional characterization of transmembrane segment IV of the NHE1 isoform of the Na^+/H^+ exchanger. J. Biol. Chem. **280**(18): 17863–17872. doi:10.1074/jbc.M409608200. PMID:15677483.

Tanaka, H., Shimada, H., Namekata, I., Kawanishi, T., Iida-Tanaka, N., and Shigenobu, K. 2007. Involvement of the Na^+/Ca^{2+} exchanger in ouabain-induced inotropy and arrhythmogenesis in guinea-pig myocardium as revealed by SEA0400. J. Pharmacol. Sci. **103**(2): 241–246. doi:10.1254/jphs.FP0060911. PMID:17310075.

van Borren, M.M., Baartscheer, A., Wilders, R., and Ravesloot, J.H. 2004. NHE-1 and NBC during pseudo-ischemia/reperfusion in rabbit ventricular myocytes. J. Mol. Cell. Cardiol. **37**(2): 567–577. doi:10.1016/j.yjmcc.2004.05.017. PMID:15276026.

Wang, Y., Meyer, J.W., Ashraf, M., and Shull, G.E. 2003. Mice with a null mutation in the NHE1 Na^+-H^+ exchanger are resistant to cardiac ischemia-reperfusion injury. Circ. Res. **93**(8): 776–782. doi:10.1161/01.RES.0000094746.24774.DC. PMID:12970112.

Wei, G.Z., Zhou, J.J., Wang, B., Wu, F., Bi, H., Wang, Y.M., et al. 2007. Diastolic Ca^{2+} overload caused by Na^+/Ca^{2+} exchanger during the first minutes of reperfusion results in continued myocardial stunning. Eur. J. Pharmacol. **572**(1): 1–11. doi:10.1016/j.ejphar.2007.05.065. PMID:17822695.

Yokoyama, H., Gunasegaram, S., Harding, S.E., and Avkiran, M. 2000. Sarcolemmal Na^+/H^+ exchanger activity and expression in human ventricular myocardium. J. Am. Coll. Cardiol. **36**(2): 534–540. doi:10.1016/S0735-1097(00)00730-0. PMID:10933369.

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