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Original article

Elevated levels of activated NHE1 protect the myocardium and improve metabolism following ischemia/reperfusion injury

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ABSTRACT

In the myocardium, the Na^+/H^+ exchanger isoform 1 (NHE1) is a plasma membrane protein that regulates intracellular pH. Inhibition of NHE1 activity has been shown to be beneficial in cardiovascular disease. However, recent reports have suggested that elevation of NHE1 levels has beneficial effects in hearts subjected to ischemia/reperfusion. We determined if activated and non-activated NHE1 proteins have varying cardioprotective and metabolic effects with ischemia/reperfusion in the isolated perfused working mouse heart. We used transgenic mice hearts that specifically expressed wild type NHE1 (N-line) or activated NHE1 protein (K-line). Intact hearts 10–12 weeks of age were perfused under working conditions, with fatty acids and glucose present as substrates. Hearts were subjected to 30 min of aerobic perfusion, followed by 20 min of global no-flow ischemia and 40 min of aerobic reperfusion. We examined changes in contractility and substrate use and ATP levels. K-line hearts expressing activated NHE1, recovered to a much greater extent than N-line and control hearts recovering almost 75% of their preischemic function. In addition, K-line hearts had elevated fatty acid oxidation, increased glycolysis rates and elevated ATP levels relative to N-line mice or controls. An examination of kinase activation showed that there were no differences between controls and transgenics in ERK, p38, $p90^{rsk}$ or pGSK3β levels. The results demonstrate that elevated levels of NHE1 induce cardioprotection and alter cardiac metabolism. However, in the working heart model, with glucose and fatty acid as substrates, this required an activated NHE1 protein.

1. Introduction

The Na^+/H^+ exchanger (NHE) is a ubiquitously expressed plasma membrane glycoprotein, which extrudes one intracellular H^+ in exchange for one extracellular Na^+ and thus regulates intracellular pH (pH_i) [1]. NHE1 (NHE, isoform 1), the only cardio-specific plasma membrane isoform [2,3], has been implicated in mediating the damage that occurs during ischemia/reperfusion injury (I/R injury) [4–7]. In numerous *in vitro* and *in vivo* studies, NHE1 inhibitors have been shown to prevent or reduce I/R injury [4,7].

NHE1 protein expression and activity increase in response to various stimuli including acidosis [8,9] and cardiac hypertrophy [10]. Interestingly, human sarcolemmal NHE1 activity was elevated in recipient hearts with chronic end stage heart failure, even though protein abundance was not increased [11], suggesting that regulation of the activity of the protein was affected. The critical physiological role of NHE1 necessitates an understanding of the effects of this varying expression and has encouraged the development of transgenic mouse models expressing altered NHE1 levels and activity. Initial studies used transgenic mice in which NHE1 was genetically ablated and demonstrated cardioprotective effects against I/R injury [12]. This is in agreement with various animal models subjected to I/R in the presence of NHE1 inhibitors. However, recent reports on transgenic mice expressing elevated NHE1 levels have demonstrated surprising results. They have shown that elevated NHE1 expression in transgenic mice can reduce the susceptibility to I/R injury [13,14]. One of these reports [13] suggested that in transgenic mice with elevated NHE1 there is a minor improvement in recovery of pH_i and ATP levels, which contributes to the cardioprotective effects. However, the cardiac energy metabolism of hearts with varying NHE1 levels has not been studied in detail.

Variation in substrate utilization occurs during I/R, and cardiac energy substrate preference has a significant impact on both contractile function and efficiency of O_2 use, particularly following severe ischemia [15,16]. In previous studies, the effects of varying NHE1 levels were examined in hearts perfused with only glucose as a substrate [13,14]. However, this is not representative of physiological substrate use by the myocardium. When glucose is the only exogenous substrate available, its utilization cannot produce more than 70% of the acetyl coenzyme A oxidized by the citric acid cycle [17]. Therefore, glycolysis does not appear to be capable of supplying adequate amounts of acetyl CoA for oxidative metabolism and for

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maintenance of tissue levels of citric acid cycle intermediates [18]. In addition, fatty acids are the main source of ATP production in normal hearts and during reperfusion following ischemia [19].

The purpose of this study is to examine cardiac energy metabolism and function in NHE1 transgenic hearts which are perfused under more physiological conditions. We have developed transgenic mice which express cardiac-specific wild type NHE1 (N-line) or constitutively activate NHE1 (K-line) [13,20]. We subjected these hearts to *ex vivo* working heart perfusion in the presence of fatty acids [21]. Rates of glycolysis, glucose oxidation and fatty acid oxidation were directly measured in both the preischemic and postischemic periods. Our results demonstrated that increased expression of active NHE1 protein is cardioprotective in the myocardium. This was accompanied by increased fatty acid oxidation, glycolysis and ATP production rates and a more energetically efficient myocardium.

2. Materials and methods

2.1. Methods (details are provided in a Supplemental section)

2.1.1. NHE1 transgenic mice

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). All experimental procedures involving animals were in accordance with guidelines set out by the Canadian Council on Animal Care. The local animal care board approved all procedures. The generation of transgenic mice overexpressing cardiac specific hemagglutinin (HA) tagged human NHE1 has been described previously [13,20,22]. The N-line represents mice expressing cardiac specific wild type NHE1 and the K-line represents mice expressing cardiac specific active NHE1. Briefly, the active NHE1-HA was made by mutating amino acids lysine 641, arginine 643, arginine 645, and arginine 647 to glutamate [23]. Mutation of this CaM binding site stimulates NHE1 activity and mimics the effects of elevated $[Ca^{2+}]_i$. This occurs because in the absence of CaM bound to Ca^{2+}, the high affinity CaM binding site causes autoinhibition of NHE1 [24]. The mutation eliminates autoinhibition and results in an NHE1 protein that is alkaline shifted in its pH dependence. We demonstrated earlier that NHE1 activity in response to acute acid load, was increased approximately two and three-fold in cardiomyocytes from N-line and K-line mice, respectively [13]. Female mice, 10–12 weeks of age were used.

2.1.2. Heart perfusions

Hearts from control and NHE1 transgenic mice were perfused using an *ex vivo* working model as described previously [25,26]. Excised hearts were immediately immersed in ice-cold Krebs–Henseleit bicarbonate (KHB) solution containing mM (118.5 NaCl, 25 $NaHCO_3$ 4.7 KCl, 1.2 $MgSO_4$ 1.2 KH_2PO_4 2.5 $CaCl_2$, 0.5 EDTA, 5 glucose, 1.2 palmitate bound to 3% fatty acid-free bovine serum albumin) and 100 μU/mL insulin. The aorta and pulmonary vein were cannulated and hearts were perfused with KHB containing either [5–^{3}H/U–^{14}C] labeled glucose and unlabeled palmitate for glycolysis and glucose oxidation measurements, or [U–^{14}C] labeled glucose and [9,10–^{3}H] labeled palmitate for glucose and palmitate oxidation measurements. Hearts were perfused aerobically for 30 min and then subjected to 20 min of global no-flow ischemia followed by 40 min of aerobic reperfusion. Pressure and flow measurements were obtained every 10 min and a 2.5 mL sample of perfusate was taken every 10 min for determination of glycolysis, glucose and palmitate oxidation. At the end of the experiment, hearts were frozen in liquid nitrogen and stored at −80 °C. To correct for variations in heart size, dry/wet weight ratio was measured. Metabolic rate production was normalized to the total dry mass of the heart, calculated from the dry/wet weight ratio.

2.2. Measurement of cardiac function and metabolism

Cardiac function was measured as described previously [27,28]. Glycolytic rates, glucose oxidation, palmitate oxidation and ATP production rates were measured as described previously [28]. Metabolic rates were compared after normalization for differences in contractile performance (cardiac power) and were divided by cardiac power [(nmol × g dry $wt^{-1} \times min^{-1}$)/(mL × mm Hg/min)]. Cardiac efficiency was determined by dividing cardiac power by ATP production rates or TCA cycle acetyl CoA production rates [(mL × mm Hg/min)/(μmol × g dry $wt^{-1} \times min^{-1}$)]. H^+ production was derived from the rate of glucose oxidation and glycolysis as described previously [21,29].

2.2.1. Western blot analyses

Heart homogenates were used for western blotting as described previously [30]. The Odyssey® Infrared Imaging System was used to visualize and quantify immunoreactive proteins (LI-COR Biosciences, Lincoln, NE).

3. Results

3.1. Preischemic and postischemic cardiac function in ex vivo working NHE1 transgenic mouse hearts

To assess the functional consequences of elevated NHE1 expression, cardiac function was measured in control, N-line and K-line mouse hearts subjected to *ex vivo* aerobic perfusion for 30 min. Initially, during aerobic perfusion, N-line and K-line hearts displayed no significant differences in mechanical function compared to controls (72 ± 4.2 controls vs. 64 ± 4.1 N-line vs. 66 ± 4.1 K-line mL × mm Hg/min, Fig. 1). Similarly, no significant differences existed in cardiac output, aortic outflow, coronary flow and cardiac work between control, N-line and K-line hearts (Table 1).

When hearts were subjected to 20 min of global no flow ischemia followed by 40 min of reperfusion, K-line hearts recovered to a much greater extent than N-line and control hearts (49 ± 4.6 K-line vs. 12 ± 4.3 N-line vs. 9.7 ± 3.6 controls mL × mm Hg/min, $P<0.0001$) (Fig. 1). K-line hearts recovered to almost 75% of their preischemic values and mechanical function was significantly improved in all parameters measured (Table 1).

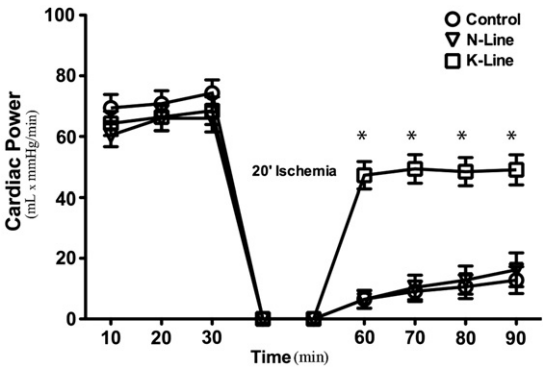

Fig. 1. Effects of elevated NHE1 expression on cardiac function in *ex vivo* aerobically perfused and reperfused working mouse heart. (N-line mice express wild type NHE1 and K-line mice express active NHE1). Hearts were subjected to 30 min of aerobic perfusion followed by 20 min of global no-flow ischemia and 40 min of aerobic reperfusion. Cardiac power, an indicator of cardiac function is shown. It was calculated from the product of developed pressure (mmHg) and cardiac output (mL/min). Values are mean ± SEM ($n = 10$–14 hearts/group). *$P<0.0001$ for control or N-line vs. K-line.

Table 1
Parameters of cardiac function in *ex vivo* working mouse hearts of NHE1 transgenic mice during aerobic perfusion (preischemia) and reperfusion (postischemia).

	Preischemia			Postischemia		
	Control	N-line	K-line	Control	N-line	K-line
Cardiac output, mL/min	8.4 ± 0.5	8.4 ± 0.4	8.4 ± 0.5	1.6 ± 0.4$^{\diamond}$	1.8 ± 0.6$^{\diamond}$	6.3 ± 0.5*
Aortic outflow, mL/min	5.7 ± 0.3	6.7 ± 0.5	6.3 ± 0.3	0.4 ± 0.2$^{\diamond}$	0.8 ± 0.5$^{\diamond}$	3.6 ± 0.4$^{\diamond}$
Coronary flow, mL/min	2.6 ± 0.3	2.0 ± 0.5	2.2 ± 0.2	1.2 ± 0.3†	1.0 ± 0.3	2.7 ± 0.3
Cardiac work, mLx mm Hg/min	6.3 ± 0.4	5.8 ± 0.4	6.0 ± 0.4	0.9 ± 0.3$^{\diamond}$	1.1 ± 0.4$^{\diamond}$	4.4 ± 0.4*

Mean average values measured over a period of 30 min (preischemia) and over a period of 40 min following 20 min of ischemia (postischemia). Values represent means ± SEM. $n = 12–16$ hearts/group. Cardiac work (mL × mmHg/min) = peak systolic pressure × cardiac output; Coronary flow = cardiac output − aortic flow. Significantly different from the corresponding preischemic group ($^{\diamond}P<0.0001$, $^{*}P<0.05$, $^{\dagger}P<0.005$).

3.2. Preischemic and postischemic rates of glycolysis, palmitate and glucose oxidation in ex vivo working NHE1 transgenic mouse hearts

When comparing control, N-line and K-line hearts, significant changes in cardiac energy metabolism were evident during preischemia and postischemia (Fig. 2). Preischemia, glucose oxidation was significantly decreased in N-line hearts vs. control (1184 ± 196 N-line vs. 2058 ± 247 control nmol × g dry $wt^{-1} \times min^{-1}$, $P<0.05$) (Fig. 2A). In K-line hearts, glucose oxidation showed a trend towards decrease vs. controls (1469 ± 179 K-line vs. 2058 ± 247 controls nmol × g dry $wt^{-1} \times min^{-1}$), but the change was not significant. During postischemia, glucose oxidation rates were not significantly different between control, N-line and K-line hearts. However, the rates of glucose oxidation during postischemia were depressed in control and N-line hearts vs. preischemic values ($P<0.05$, Fig. 2A). In contrast, glucose oxidation was not significantly altered in preischemic K-line hearts vs. postischemic K-line hearts.

During preischemia, palmitate oxidation rates were comparable between control and N-line hearts (Fig. 2B). However, K-line hearts had a significantly greater rate of palmitate oxidation vs. controls (380 ± 31 controls vs. 513 ± 26 K-line nmol × g dry $wt^{-1} \times min^{-1}$, $P<0.05$, Fig. 2B). Palmitate oxidation remained significantly greater in K-line hearts during postischemia (215 ± 37 control vs. 399 ± 21 K-line nmol × g dry $wt^{-1} \times min^{-1}$, $P<0.05$, Fig. 2B). When

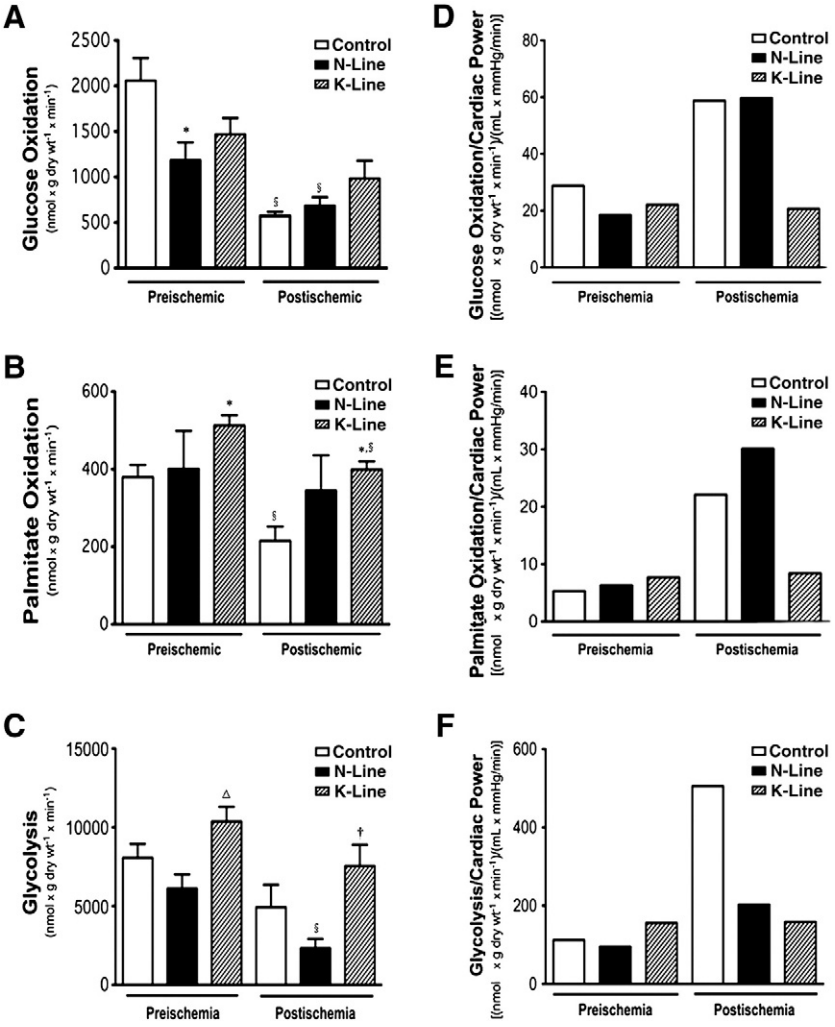

Fig. 2. Rates of glucose oxidation (A), palmitate oxidation (B), and glycolysis (C) in control, (wild type NHE1) N-line and (active NHE1) K-line hearts during preischemic and postischemic periods. Preischemic values were obtained during 30 min of aerobic perfusion. Postischemic values were obtained following 20 min of global no flow ischemia and during 40 min of aerobic reperfusion. Glucose oxidation (D), palmitate oxidation (E), and glycolysis (F) were normalized for cardiac power. Values represent the mean ± SEM ($n = 5–10$ hearts/group). For corresponding control, $^{*}P<0.05$ for control vs. N-line or K-line and $^{\Delta}P<0.05$, $^{\dagger}P<0.01$ for N-line vs. K-line. $^{\S}P<0.05$ for preischemic vs. postischemic.

compared to preischemic values, the postischemic levels of palmitate oxidation were significantly depressed for both control and K-line hearts ($P<0.05$, Fig. 2B). In N-line hearts, palmitate oxidation decreased slightly vs. preischemic values, but the changes were not significant.

Preischemic levels of glycolysis were significantly elevated in K-line *ex vivo* working hearts vs. N-line hearts (8065 ± 876 controls vs. 6105 ± 905 N-line vs. $10{,}366 \pm 936$ K-line nmol × g dry $wt^{-1} \times min^{-1}$, $P<0.05$, Fig. 2C). There was no significant difference between N-line and control hearts in preischemic levels of glycolysis. During the postischemic reperfusion period, glycolytic rates remained significantly elevated in the K-line mouse hearts compared to N-line (2326 ± 595 N-line vs. 7538 ± 1352 K-line nmol × g dry $wt^{-1} \times min^{-1}$, $P<0.01$, Fig. 2C). In N-line hearts postischemia, glycolysis rates were not significantly different than control hearts, but were significantly depressed vs. preischemic values (6105 ± 905 preischemia vs. 2326 ± 595 postischemia nmol × g dry $wt^{-1} \times min^{-1}$, $P<0.05$, Fig. 2C).

Since cardiac work is an important determinant of metabolic rates, glucose oxidation and palmitate oxidation and glycolysis were normalized for cardiac power (Figs. 2D–F). During preischemia, there was little difference in cardiac power (Fig. 1) and thus the rates of glucose and palmitate oxidation and glycolysis, varied little when normalized for function. However, mechanical function during postischemia varied notably and was elevated in K-line hearts (Fig. 1). With correction, it was notable that in K-line hearts both glucose oxidation and palmitate oxidation relative to cardiac power were greatly decreased. The same trend occurred for both N-line and K-line hearts with regards to glycolysis. Preischemic vs. postischemic rates of palmitate oxidation, glucose oxidation and glycolysis normalized to cardiac power were comparable in K-line hearts. In contrast, during postischemia, the rates of glucose and palmitate oxidation were elevated in control and N-line hearts vs. preischemia. Similarly, the glycolytic rates normalized for cardiac power, were elevated in control hearts when preischemic vs. postischemic rates were compared.

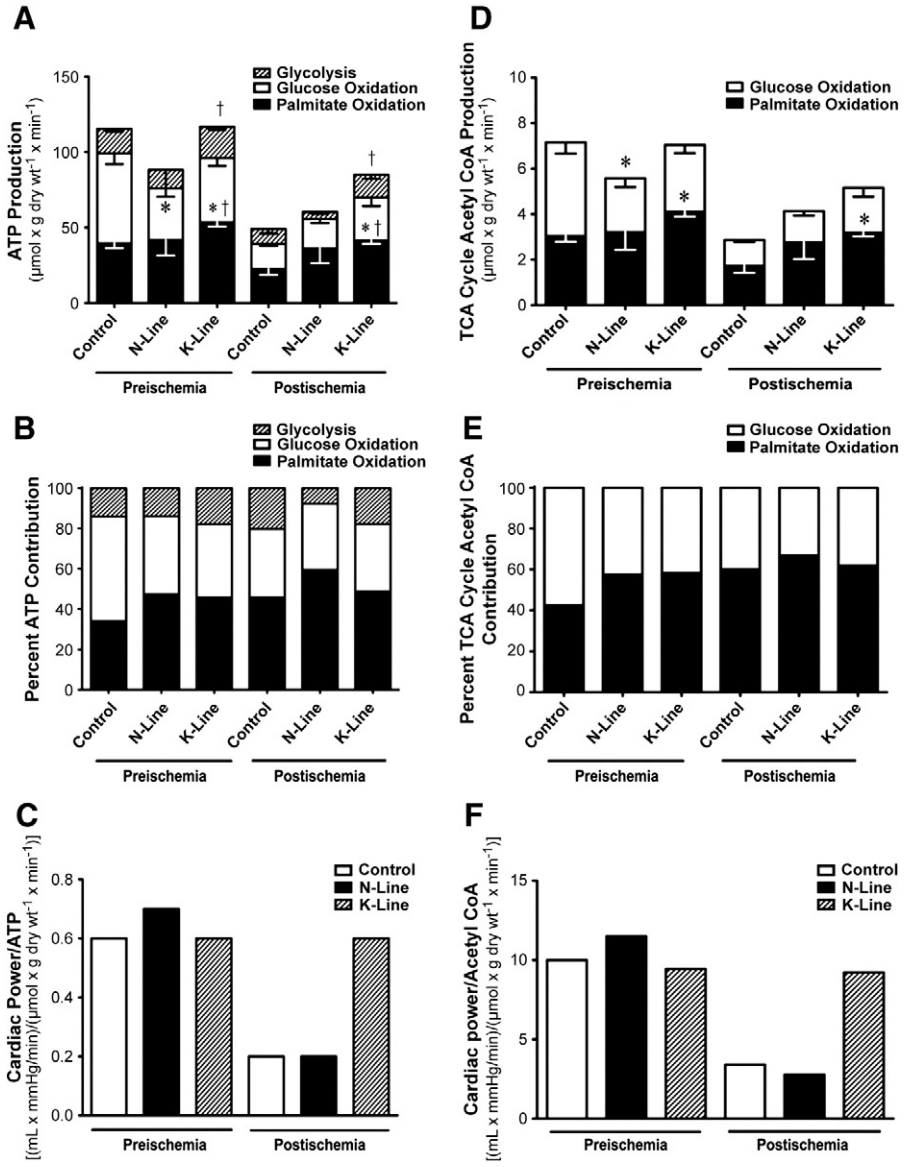

Fig. 3. Total ATP production rates and TCA cycle acetyl CoA production rates in control, and transgenic hearts during preischemic and postischemic periods. Preischemic and postischemic values were from the times indicated in Fig. 2. (A), ATP production rates were calculated from the rates of palmitate oxidation and glucose oxidation and glycolysis. (B), Percentage of ATP production rates contributed by palmitate oxidation, glucose oxidation or glycolysis. (C), Total ATP production rates normalized to cardiac power. (D), TCA cycle acetyl CoA production rates from palmitate oxidation and glucose oxidation. (E), Percentage of TCA cycle acetyl CoA contributed by palmitate oxidation or glucose oxidation. (F), TCA cycle acetyl CoA production rates normalized to cardiac power. Values are expressed as mean ± SEM ($n = 5$–10 hearts/group). *$P<0.05$ for control vs. N-line or K-line; †$P<0.05$ for N-line vs. K-line.

3.3. Preischemic and postischemic ATP production in ex vivo working NHE1 transgenic mouse hearts

We calculated the relative contribution of glycolysis, glucose oxidation and palmitate oxidation to ATP production during preischemia and postischemia (Figs. 3A–C). During preischemia, fatty acid oxidation accounted for a greater amount of energy production in K-line hearts (53.4 ± 2.7 μmol × g dry $wt^{-1} \times min^{-1}$) vs. both control (39.5 ± 3.2 μmol × g dry $wt^{-1} \times min^{-1}$) and N-line hearts (41.7 ± 10.2 μmol × g dry $wt^{-1} \times min^{-1}$) ($P<0.05$, Fig. 3A). This was part of a general trend of palmitate oxidation, contributing a greater percentage of ATP production rates and TCA cycle acetyl CoA production rates, during preischemia in NHE1 transgenic vs. control mice (Figs. 3B and E). During preischemia, ATP derived from glycolysis in K-line hearts was significantly greater vs. N-line hearts (12.2 ± 1.8 N-line vs. 20.7 ± 1.9 K-line μmol × g dry $wt^{-1} \times min^{-1}$, $P<0.05$, Fig. 3A). However, ATP derived from glucose oxidation was relatively similar in control and K-line hearts and significantly depressed in N-line hearts ($P<0.05$, Fig. 3A). Total ATP production rates were significantly greater in control and K-line hearts vs. N-line hearts, suggesting that N-line hearts are energetically compromised during preischemia. TCA cycle acetyl CoA production rates during preischemia reflected trends similar to that seen with ATP production rates. The contribution of glucose oxidation to TCA cycle acetyl CoA production rates was significantly decreased in N-line hearts ($P<0.05$, Fig. 3D). TCA cycle acetyl CoA production rates from palmitate oxidation were significantly greater in K-line hearts vs. control hearts ($P<0.05$, Fig. 3D). This resulted in greater total TCA cycle acetyl CoA production rates in control and K-line hearts vs. N-line hearts (Fig. 3D).

During postischemia palmitate predominated as the source of ATP and TCA cycle acetyl CoA production rates in all hearts (Figs. 3B and E). Also, during postischemia, total ATP production and total TCA cycle acetyl CoA production in K-line hearts was greater vs. control ($P<0.05$, Figs. 3A and D). The contribution of glucose oxidation and palmitate oxidation to TCA cycle acetyl CoA production rates and ATP production rates were similar in control and N-line hearts (Fig. 3A). However, in K-line hearts palmitate was significantly elevated as a source of acetyl CoA for the TCA cycle (1.7 ± 0.3 controls vs. 3.2 ± 0.2 K-line μmol × g dry $wt^{-1} \times min^{-1}$, $P<0.05$, Figs. 3D–E) as it was for ATP production rates (22.5 ± 3.9 controls vs. 41.4 ± 2.2 K-line, $P<0.05$, Fig. 3A). Part of the higher levels of ATP production rates in the postischemic K-line hearts were a result of glycolysis derived ATP, as this was also significantly greater in K-line hearts vs. N-line hearts (15.1 ± 2.7 K-line vs. 4.65 ± 1.2 N-line μmol × g dry $wt^{-1} \times min^{-1}$, $P<0.01$, Fig. 3A). Postischemia, a notable change was that the efficiency of energy utilization, indicated by total ATP production rates and acetyl CoA production normalized to cardiac power was greater in K-line hearts vs. control hearts (Figs. 3C and F).

3.4. Preischemic and postischemic proton production in ex vivo working NHE1 transgenic mouse hearts

We also examined the rate of H^+ production, which was derived from the degree of uncoupling of glucose oxidation and glycolysis [31]. A greater reliance on glucose as a source of mitochondrial acetyl CoA production, suggests that more pyruvate and protons are being consumed aerobically in the TCA cycle, which is not the case in N-line and K-line hearts (Fig. 3D), thus suggesting that more protons are being produced. In addition, ATP derived from glycolysis, which is significantly elevated in K-line mice (Fig. 3A), leads to the production of H^+ [21]. During preischemia, K-line hearts produced 1.5-fold more protons than control hearts (17.8 K-line vs. 12.0 control μmol × g dry $wt^{-1} \times min^{-1}$), whereas in N-line hearts, proton production remained relatively comparable to control hearts (9.84 N-line vs. 12.0 control μmol × g dry $wt^{-1} \times min^{-1}$). During postischemia reperfusion,

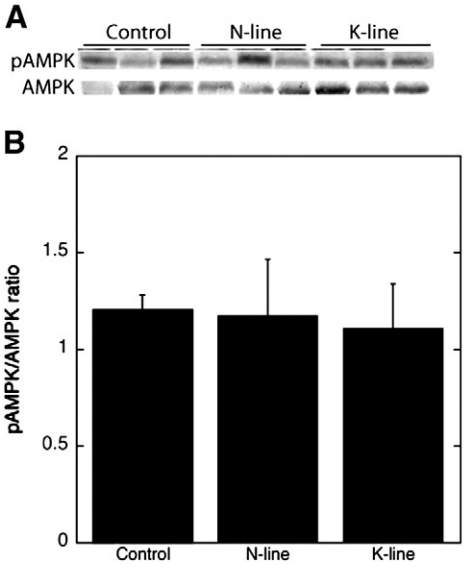

Fig. 4. Immunoblot analysis of total and phosphorylated AMPK expression in control, N-line and K-line mouse heart lysates. Hearts were subjected to 30 min of aerobic perfusion, 20 min of global no flow ischemia and 40 min of aerobic perfusion. (A), Western blot of total phosphorylated AMPK and total AMPK protein expression. (B), quantification of a series of experiments. Results are expressed as a ratio of phosphorylated AMPK to total AMPK. ($n = 5–6$ hearts/group).

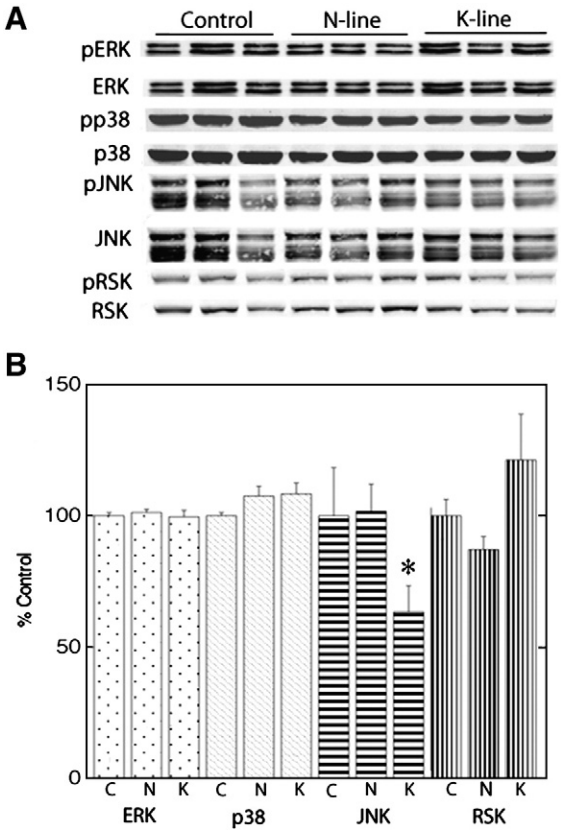

Fig. 5. Analysis of signaling pathways in control, N-line and K-line mouse heart lysates. Hearts were subjected to 30 min of aerobic perfusion, 20 min of global no flow ischemia and 40 min of aerobic reperfusion. (A), western blot of relative amounts of phosphorylated and total protein expression of ERK, p38, JNK and RSK. (B), quantification of a series of experiments measuring the ratio of phosphorylated to total protein for phospho-ERK/ERK, phospho-p38/p38, phospho-JNK/JNK and phospho-RSK/RSK. Results are expressed as a% of controls (for each group) ± SEM. ($n = 5–6$ hearts/group). *$P<0.05$ vs. control or N-line.

we found that the rates of H^+ production in K-line hearts remained elevated and was greater than proton production in control and N-line hearts (13.1 K-line vs. 8.7 controls vs. 3.3 N-line μmol × g dry $wt^{-1} \times min^{-1}$).

3.5. Signaling pathways of NHE1 transgenic hearts

We examined activation of various signaling pathways *in vivo* to determine their contribution, if any, to the NHE1-mediated cardioprotection or lack of cardioprotection following I/R injury. Initially, we examined the level of phospho-AMP-activated protein kinase (AMPK), an important regulator of myocardial energy metabolism during I/R injury [32]. Western blot analysis of phospho-AMPK protein kinase levels relative to AMPK levels indicted that there were no differences in the phosphorylation level of the protein between control, N-line and K-line hearts (Fig. 4). We than examined extracellular signal-regulated kinases, pathways that are potentially activated by I/R injury [33] (Fig. 5). The level of phospho-ERK, phospho-p38 and phospho-RSK were not elevated in transgenic hearts compared to controls (Fig. 5). However, the level of phospho-JNK, as indicated by the ratio of phospho-JNK to total JNK, was significantly attenuated in K-line hearts vs. control ($P<0.05$, Fig. 5).

Glycogen synthase kinase-3β (GSK-3β) has been suggested to play a pivotal cardioprotective role [34]. However no significant changes were evident in GSK-3β protein or phosphoprotein levels in NHE1 transgenic mouse hearts (Fig. 6).

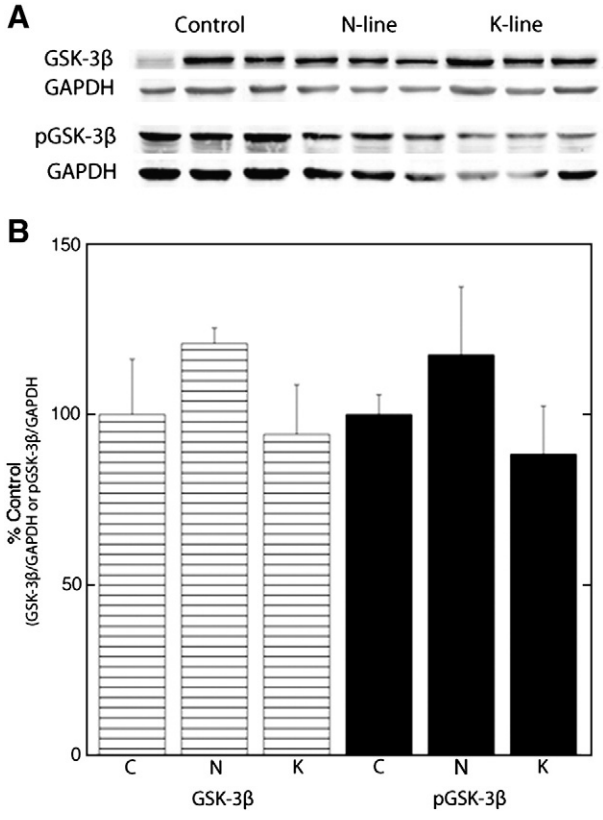

Fig. 6. Analysis of total and phosphorylated GSK-3β in control, N-line and K-line mouse heart lysates. Hearts were subjected to 30 min of aerobic perfusion, 20 min of global no flow ischemia and 40 min of aerobic reperfusion. (A), western blot of amounts of total and phosphorylated GSK-3β relative to GAPDH. (B), quantification of a series of experiments measuring the amounts of phosphorylated and total GSK-3β protein. (Results are expressed as a% of controls (for each group) ± SEM. ($n=5$–6 hearts/group).

4. Discussion

Although many studies have suggested that inhibition of NHE1 attenuates I/R injury, recent reports in two different transgenic mouse models have suggested that elevated expression of NHE1 in transgenic mice induces the opposite, cardioprotective effects, in hearts subjected to I/R injury [13,14]. The role that NHE1 plays in the cardioprotection of hearts subjected to I/R injury is still unclear. Our previous report suggested that following I/R, mice expressing elevated NHE1 demonstrated a minor improvement in recovery of pH_i and ATP [13]. This preliminary observation led to the more detailed examination of cardiac metabolism in aerobically perfused and I/R hearts from mice expressing various types of NHE1 protein.

Because we wanted to examine cardiac energy metabolism in more detail and under more physiological conditions, we used the *ex vivo* isolated working heart model with the inclusion of fatty acid as a substrate. This model was used because it has a higher and more physiological energy demand compared with Langendorff perfused hearts [35], which were used earlier [13,14]. In addition, the Krebs–Henseleit (KHB) perfusate used in this model contained both glucose and fatty acids as a substrate making it more representative of normal human plasma in the setting of ischemia [21]. Palmitate is the preferred energy substrate of the normal perfused heart [16] and is used by the myocardium in reperfusion following ischemia [21]. In our earlier study we found that ischemia causes a rapid and large drop in intracellular pH that was equivalent in both wild type and transgenic animals [13]. Similarly, studies by Murphy and coworkers have shown precipitous drops in intracellular pH with ischemia in isolated perfused hearts [36,37]. In the present study we did not monitor intracellular pH of the isolated perfused hearts so it is possible that in contrast to earlier observations [13], intracellular pH in hearts with activated NHE1 declined to a lesser degree than in the other groups, though this possibility seems unlikely. In this regard it is of interest that the decline in contractility during ischemia was similar in all groups (Fig. 1).

Table 2
Summary of relative values of metabolic and functional parameters of control and transgenic mice.

Parameter	Control	N-Line	K-Line
A. Preischemia			
Cardiac power	+++++	+++++	+++++
Cardiac output	+++++	+++++	+++++
Aortic outflow	+++++	+++++	+++++
Coronary flow	+++++	+++++	+++++
Cardiac work	+++++	+++++	+++++
Glucose oxidation	+++++	+++	++++
Palmitate oxidation	++++	++++	+++++
Glycolysis	++++	+++	+++++
Glucose oxidation/cardiac power	+++	++	++
Palmitate oxidation/cardiac power	+	++	++
Glycolysis/cardiac power	++	+	++
Total ATP production	+++++	++++	+++++
Cardiac power/ATP	+++++	+++++	+++++
Cardiac power/acetyl CoA	+++++	+++++	+++++
B. Postischemia			
Cardiac power	++	++	++++
Cardiac output	++	++	++++
Aortic outflow	+	+	+++
Coronary flow	++	++	+++++
Cardiac work	+	+	++++
Glucose oxidation	+	++	+++
Palmitate oxidation	++	+++	++++
Glycolysis	+++	++	++++
Glucose oxidation/cardiac power	+++++	+++++	++
Palmitate oxidation/cardiac power	++++	+++++	++
Glycolysis/cardiac power	+++++	++	++
Total ATP production	++	+++	++++
Cardiac power/ATP	++	++	+++++
Cardiac power/acetyl CoA	++	++	+++++

A number of important findings were made (summarized in Table 2). Firstly, similar to our previous results [13] elevated cardiac specific expression of wild type or activated NHE1 does not functionally compromise the heart function during aerobic perfusion. On the contrary, we found that expression of NHE1 during I/R produced a marked cardioprotection during reperfusion of the ischemic hearts. However, in the present study, a novel finding was that only activated NHE1 (K-line) was cardioprotective while earlier [13] we found that cardioprotection occurred with both N-line and K-line mouse hearts. The difference between this and the previous report is likely due to the difference in the perfusion conditions. Either inclusion of fatty acid or the greater workload of the present study was likely accountable. While our results agree with previous work that showed that elevated expression of NHE1 can be cardioprotective in the myocardium, we found that under our present conditions, expression of wild type NHE1 was not beneficial and increased activity of the protein was required for cardioprotection.

Why would addition of a constitutively active NHE1 protein be more effective at cardioprotection in comparison with increased levels of the non-activated protein? The NHE1 protein is regulated by a proton sensor such that at more alkaline pH's, the activity is minimal. Activation of the protein shifts the pH dependence to a more alkaline range [2]. It may be that increased levels of the wild type protein provides minimal benefit if the protein is not activated. Shifting the activity to the more alkaline range, with the K-line mutation [13], may provide a more functional protein at critical pH's.

To gain further insights into the mechanism of NHE1 mediated cardioprotection, we examined cardiac energy metabolism and signaling pathways. The notable metabolic differences in K-line hearts in comparison to the control and N-line hearts were: (1) an elevation in fatty acid oxidation, preischemia and postischemia; (2), an elevation in glycolysis, preischemia and postischemia, (3), a lack of postischemia reduction in glucose oxidation and (4) significantly greater levels of total ATP production rates and TCA cycle derived acetyl CoA production rates (Table 2).

Recently, approaches to the treatment of cardiovascular disease have concentrated on minimization of fatty acids as a fuel, while increasing glucose oxidation. This is believed to minimize the degree of acidosis associated with I/R injury [38,39]. However, in our study, we found that palmitate oxidation was elevated in K-line relative to N-line and control hearts, and this occurred with an improvement in contractile function in K-line mice. While the mechanism by which this occurred is uncertain, it could reflect the increased activity of the K-line NHE1 protein, which could aid in removal of excess acid and minimize acidosis.

In K-line *ex vivo* working hearts, glycolysis and glucose oxidation were both significantly elevated during both preischemic and postischemic periods, which contributed to higher rates of ATP production. Increasing glucose oxidation has been shown to have a beneficial effect on functional recovery of hearts [40]. Elevated glycolysis can also be protective during and following myocardial ischemia [41]. However, if glycolysis is increased to a greater extent than glucose oxidation, an increased proton load on the heart can occur, which can contribute to a decrease in cardiac efficiency and a decrease in functional recovery postischemia [15]. Indeed, in K-line hearts, proton production from uncoupled glycolysis to glucose oxidation was actually increased. Despite this, these hearts showed the best recovery of function postischemia. Opie [42] has suggested that enhanced glycolysis is more beneficial to the myocardium when pH_i is maintained appropriately. It maybe that elevated activity of the K-line NHE1 protein, coupled with enhanced but "pH-managed" glycolysis, were responsible for at least part of the protective effects that were observed. While the K-line hearts did show greater levels of proton production, we earlier [13] demonstrated that transgenic hearts with elevated NHE1 were able to maintain pH as well as the other hearts, and following reperfusion minor improvements in pH_i recovery were evident. This data challenges the widely held belief that excessive NHE1 activity contributes to ischemic injury, but rather suggests that maintenance of pH is more important in postischemic recovery.

We also examined a number of signaling pathways and stress related proteins in this experimental model. Numerous signaling pathways are activated during I/R injury, contributing to either I/R injury or protecting the heart from further injury. In this study, we determined whether such pathways contribute to the cardioprotective effect seen in mice expressing elevated NHE1. Initially, we investigated AMPK, an important regulator of myocardial energy metabolism during I/R injury [43]. AMPK has been shown to be rapidly activated during myocardial ischemia and is associated with an increase in fatty acid oxidation as well as an increase in glycolysis [44,45]. However, no differences were seen in N-line and K-line hearts vs. controls, which suggests that AMPK was not responsible for the increases in these metabolic rates.

We additionally investigated the primary protein kinase pathways activated by myocardial I/R, which include the MAPKs, ERK 1/2, JNK 1/2, p38 MAPK and RSK [33]. No significant differences were seen between control and NHE1 transgenic hearts in activation of ERK 1/2, p38 MAPK and RSK. However, JNK phosphoprotein levels significantly decreased in K-line hearts vs. control and N-line hearts. JNK is activated in response to various cellular stresses including inflammatory cytokines, ischemia, reversible ATP depletion, heat shock, endotoxin, and genotoxic stress [46]. The decrease in JNK phosphoprotein levels in I/R-treated hearts provides further evidence that our K-line hearts were not as energetically or functionally stressed as the controls and N-line hearts.

Another kinase we examined was GSK-3β, which has been suggested to be crucial in cardioprotection during I/R [34]. In our study, no differences were seen between GSK-3β protein expression and phosphorylation in control, N-line and K-line hearts suggesting that the cardioprotection seen in K-line hearts is not mediated through this mechanism. It should be noted however that with GSK-3β and the other protein kinases, we only examined the phosphorylation levels at one time point, 40 min after reperfusion. While we have found earlier that these kinases can be activated by a similar protocol in rat hearts [47] it is possible that earlier or later differences in activation of the kinases may occur.

We recently [48] examined changes in gene expression that occur in N- and K-line hearts. K-lines hearts in particular, had a varied pattern of gene expression that led to hypertrophy. Cook et al. [14] have shown that some ER stress proteins are elevated in NHE1 overexpressing mice. We [48] found an elevation in calreticulin and some isoforms of heat

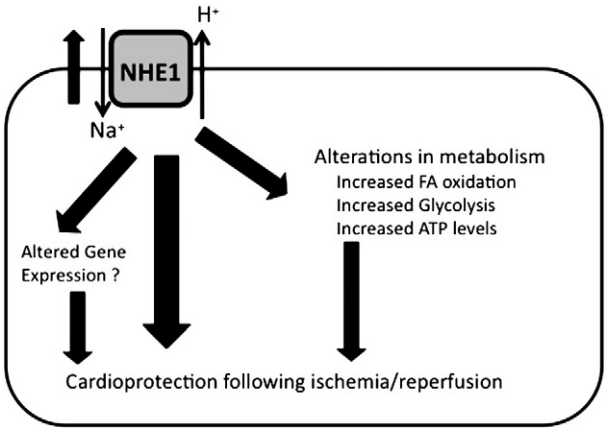

Fig. 7. Schematic representation of the role of activated NHE1 expression during ischemia reperfusion. Expression of active NHE1 in transgenic mice induces cardioprotection in hearts subjected to ischemia/reperfusion. This may occur directly through enhanced proton extrusion, through improvements in cardiac metabolism, or secondarily through alterations in gene expression.

shock protein in K-line mice, though it is uncertain at this time whether they contribute to cardioprotection in this model.

5. Conclusion

Overall, our results show that elevated expression of constitutively active NHE1 protein results in cardioprotection of the myocardium, with improvement in a number of functional parameters postischemia. Fig. 7 illustrates a summary of our results and some hypothetical relationships. Increased levels of the NHE1 may be directly beneficial in coping with proton production that occurs with elevated use of fatty acids and glycolysis. This could lead to cardioprotection. Increased NHE1 activity may act indirectly through alteration of expression of other proteins to cause cardioprotection. In addition, increases in NHE1 activity caused increased fatty acid oxidation, glycolysis and ATP levels which may lead to cardioprotection, especially when coupled with elevated proton extrusion. Future experiments could examine other models of heart disease such as if elevated NHE1 activity could affect infarct size.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.yjmcc.2010.10.016.

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