Basic Res Cardiol (2011) 106:603–616
DOI 10.1007/s00395-011-0161-4

ORIGINAL CONTRIBUTION

Activated NHE1 is required to induce early cardiac hypertrophy in mice

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Received: 26 September 2010 / Revised: 7 December 2010 / Accepted: 13 December 2010 / Published online: 27 February 2011

Abstract The Na^+/H^+ exchanger isoform 1 (NHE1) has been implicated as being causal in cardiac hypertrophy and the protein level and activity are elevated in the diseased myocardium. However, it is unclear whether mere elevation of the protein is sufficient for cardiac pathology, or whether activation of the protein is required. In this study, we examined the comparative effects of elevation of wild type and activated NHE1. Two mouse transgenic models that expressed either a wild type NHE1 protein or an activated NHE1 protein were characterized. Expression of activated NHE1 caused significant increases in heart weight to body weight, apoptosis, cross-sectional area, interstitial fibrosis and decreased cardiac performance. Expression of wild type NHE1 caused a much milder pathology. When we examined 2 or 10-week-old mouse hearts, there was neither elevation of calcineurin levels nor increased phosphorylation of ERK or p38 in either NHE1 transgenic mouse line. Expression of activated NHE1 in intact mice caused an increased sensitivity to phenylephrine-induced hypertrophy. Our results show that expression of activated NHE1 promotes cardiac hypertrophy to a much greater degree than elevated levels of wild type NHE1 alone. In addition, expression of activated NHE1 promotes greater sensitivity to neurohormonal stimulation. The results suggest that activation of NHE1 is a key component that accentuates NHE1-induced myocardial pathology.

Keywords Cardiomyocyte · Cardiac hypertrophy · Na^+/H^+ exchanger · Phenylephrine

Abbreviations

AIF	Apoptosis inducing factor
NHE	Na^+/H^+ exchanger
HA	Hemagglutinin
PARP	Poly (ADP-ribose) polymerase

Introduction

The Na^+/H^+ exchanger (NHE) is a ubiquitously expressed plasma membrane glycoprotein. One of its key functions is the regulation of intracellular pH by extruding one intracellular H^+ in exchange for one extracellular Na^+ [25]. NHE isoform 1 (NHE1) is the only cardio-specific plasma membrane isoform of the ten isoforms described [10, 24, 45]. NHE1 plays a critical role in mediating the damage that occurs during ischemia/reperfusion injury (I/R injury) [2, 23, 39] and is an important mediator of cardiac hypertrophy [40]. Recent studies have shown that NHE1 activity is increased in in vivo and in vitro models of cardiac hypertrophy [21, 38] and NHE1 inhibition is beneficial in the prevention/regression of several models of myocardial hypertrophy [7, 20, 21, 38, 47, 64]. However,

Electronic supplementary material The online version of this article (doi:10.1007/s00395-011-0161-4) contains supplementary material, which is available to authorized users.

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the direct role of NHE in cardiac hypertrophy and the effects of elevation of NHE1 activity and expression, both in vivo and in vitro, have not been fully defined.

Elevation of NHE1 expression and activity occurs in several cardiac pathologies [19, 21, 27, 38, 62]. However, NHE1 is regulated such that it is active at acidic pH's but inactive at physiological pH's [14]. Hormonal stimulation of NHE1 activity changes the set point so that the protein is more active at more alkaline pH's [14, 45]. This may occur through phosphorylation mediated by extracellular signal-regulated kinases [22] that are activated in heart failure and are attenuated by NHE-inhibition that reduces derangements induced in heart failure models [1]. Because the unstimulated NHE1 protein is relatively quiescent at resting physiological pH's [14], it is questionable whether elevation of a non-activated protein alone is detrimental to the myocardium. In this study, we examined the role that the activated NHE1 protein has in causing cardiac hypertrophy/damage relative to the non-activated protein. We characterized two models over-expressing NHE1, one expressing an activated form of NHE1 and the other expressing a non-activated NHE1. This was done in transgenic mice. We demonstrate that elevated NHE1 expression with elevated activity potentiates cardiac hypertrophy and causes hypersensitivity to neurohormonal stimulation, while mere elevation of NHE1 protein has much less significant effects. Our results demonstrate that enhanced NHE1 expression alone causes little cardiac pathology, but that enhanced NHE1 activity is critical to induce detrimental effects in the myocardium.

Materials and methods

Materials

Primary antibodies used in this study were anti-HA tag (Y-11), anti-Actin (H-300), p^{90} ribosomal s6 kinase (RSK) 1 (C21) (s-231) and 2 (C18) (sc-1430), p38-mitogen activated protein kinase (N-20) (sc-728), phospho-p38 ($Thr^{180/182}$) (sc-17852), p38-mitogen activated protein kinase α (C-20) (sc-535) and c-Jun N-terminal kinase (JNK) (FL) (sc-571) and pJNK (Thr^{183}/Tyr^{185}) (sc-12882), all from Santa Cruz Biotechnology (Santa Cruz, Ca). Anti-$Na^{+/}H^{+}$ exchanger isoform 1 was from BD Biosciences Pharmingen (San Diego, CA, USA). Extracellular signal-regulated kinase (ERK)1/2 (#9102), phospho-ERK1/2 (Thr^{202}/Tyr^{204}) (#9106), phospho-RSK (Ser^{380})(#9323), apoptosis-inducing factor (AIF) (#4642), cytochrome c (Cyt c) (#4272), poly (ADP-ribose) polymerase (PARP) (#9542), glycogen synthase kinase-3β (GSK-3β) (27C10) and phospho-GSK-3β (Ser9) (5B3) were from Cell Signaling Technology (Beverly, MA, USA). Anti-HA (12CA5) was from Roche Applied Science (Laval, Quebec, Canada). Mitochondrial superoxide dismutase (MnSOD) (SOD-110) was from Stressgen (Ann Arbor, MI, USA). Histone (H5100-02B) was from US Biological. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (ab9485) and ß-tubulin (ab6046) were from Abcam (Cambridge, MA, USA). Anti-calreticulin and protein disulfide isomerase (PDI) were a generous gift from Dr. Marek Michalak at the University of Alberta (Edmonton, Alberta, Canada). Secondary antibodies of specific wavelength were purchased from LI-COR Biosciences (Lincoln, NE, USA). Hoescht (33258) was from Sigma-Aldrich, St. Louis, MO, USA.

NHE1 transgenic mice

All experimental procedures involving animals presented in this study were used in accordance with the guidelines set out by the Canadian Council on Animal Care. The generation of transgenic mice expressing hemagglutinin (HA) tagged human NHE1 under the control of the alpha myosin heavy chain promoter was described previously [12, 34]. Transgenic mice expressing wild type NHE1 protein are referred to as N-line, and K-line mice express an activated NHE1 in the myocardium. As previously described [12], several lines for each transgenic model were created. In the following study, the N-line and K-line mice expressing similar levels of NHE1 were characterized. N-line and K-line mice used in this study were 10–12 weeks of age. Littermates were used from either N-line or K-line mice as controls. In some experiments, mice were treated with either phosphate buffered saline (vehicle) or 50 mg/kg/day phenylephrine (PE) dissolved in PBS for a period of 2 weeks using Alzet osmotic mini pumps [16, 53, 58]. Mice were anesthetized with a cocktail containing a mixture of ketamine and acepromazine. Isoflurane was used for the duration of the surgery to maintain the anesthesia. A small incision was made in the mid scapular region. By spreading apart the subcutaneous tissue, a small pocket was created and the osmotic mini pump was inserted (Alzet Osmotic Pumps, Cupertino, CA, USA). The incision was closed with sutures. At the onset of treatment, drug-treated mice were paired with vehicle-treated mice. They were matched for age and body weight, and were housed together throughout the experiment. The pumps were removed 2 weeks following implantation, at which time, animals were euthanized and hearts were removed for further analysis. PE or PBS delivery through osmotic mini pumps was verified by measuring the residual volume in the osmotic mini pumps. This value corresponded with the predicted residual volume [actual fill volume − (mean pump rate × infused duration (hours))].

Western blot analysis and sample preparation

Western blotting for NHE1 protein was as described earlier [12, 34]. Hearts were homogenized in (mM) [120 NaCl, 10 Tris (pH 7.4), 0.1 PMSF, 0.1 Benzamidine] and 37.5 μM ALLN (calpain I inhibitor) and a proteinase inhibitor cocktail. Proteins were solubilized in SDS and total protein was quantified using the Bio-Rad D_C Protein Assay kit. 100 μg of each sample was resolved on 10% SDS polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were incubated with anti-HA or anti-NHE1 over night. Actin was used as a loading control. Membranes were then incubated in species and wavelength specific LI-COR secondary antibodies for 30 min. The Odyssey® Infrared Imaging System was used to visualize and quantify immunoreactive proteins (LI-COR Biosciences, Lincoln, NE, USA). For pERK/ERK, pp38/p38(α), pJNK/JNK and pRSK/RSK immunoblots, heart tissue was homogenized at a high setting with an OMNI 2000 homogenizer (OMNI International, Kennesaw, GA, USA) in lysis buffer containing (mM) [50 HEPES, 100 NaCl, 50 NaF, 1 EGTA, 1 EDTA, 1% Triton X-100, 4 PMSF, 0.8 Na_3VO_4 and μM (0.6 aprotinin, 8.6 leupeptin, 5.8 pepstatin A]. The homogenate was centrifuged at 100,000×*g* for 10 min at 4°C and the supernatant was discarded [37]. Markers of apoptosis, including AIF and Cyt c and PARP, were measured in both the cytosolic and mitochondrial fraction. Mitochondrial and cytosolic fractions were prepared as described previously [35]. Briefly, heart tissue was homogenized in sucrose buffer containing (mM) [sucrose 300, 10 Tris–HCl, 2 EGTA; pH 7.4, 0.5% BSA] and then centrifuged at 2,000×*g* for 2 min at 4°C, followed by centrifugation of the supernatant at 10,000×*g* for 5 min at 4°C. The pellet, the mitochondrial fraction, was then washed thrice at 10,000×*g* for 5 min at 4°C and resuspended in sucrose buffer. The latter was centrifuged at 100,000×*g* for 60 min at 4°C and the supernatant was used as the cytosolic fraction. The purity of the mitochondrial fraction was tested by immunoblotting the lysate with anti-histone, a nuclear marker. Cytosolic or mitochondrial lysates immunoblotted with anti-Cyt C were resolved on 8% SDS polyacrylamide gels, whereas lysates immunoblotted with anti-AIF or anti-PARP were resolved on 10% SDS polyacrylamide gels. MnSOD was used as a loading control for the mitochondrial fraction; GAPDH was used as a loading control for cytosolic fraction. pGSK-3*β*/GSK-3*β* protein expression was also measured in the cytosolic and mitochondrial lysates.

Transgenic heart evaluation

Mice were euthanized with halothane and hearts were isolated for the following analyses, and used as markers of cardiac hypertrophy: (1) heart weight to body weight ratio (HW/BW); (2) histological study; (3) atrial natriuretic peptide mRNA expression. For HW/BW, all extracardiac structures were excised from isolated hearts and then hearts were washed in PBS, blotted and weighed [17]. Cardiac hypertrophy was evaluated by measuring HW/BW, in mg/g. To exclude the possibility that any increase in heart weight was due to edema, edema was evaluated in the isolated hearts by measuring water content. The initial weight of the isolated heart is the wet weight. Dry weight is the weight after incubation at 100°C for 24 h. The dry weight to wet weight ratio was calculated according to the formula: dry tissue weight/wet tissue weight [9].

For histology, isolated hearts were fixed overnight in 10% buffered formalin, then embedded in paraffin, and serially sectioned into 5-μm thick slices [8, 47]. These were stained with hematoxylin and eosin (H&E) to evaluate gross morphology and cross-sectional area (CSA) or with picrosirius red (PSR) to assess interstitial fibrosis (IF) [49]. IF was measured from digitized images using Open lab 3.5 (Improvision Inc., Waltham, MA, USA) and digitized images were gathered using a QImaging camera (QImaging, Surrey, BC, Canada) equipped to a Leica DMLA microscope (Leica Microsystems, Bannockburn, IL, USA). Three or four sections were used from each heart with ten fields randomly selected. The maximum fibrosis observed for any section was calculated (area occupied by red stained connective tissue) × 100/(areas occupied by connective tissue plus cardiac myocytes). Intramural vessels, perivascular collagen, endocardium and trabeculae were excluded [32]. CSA was measured from digitized images using Image ProPlus Version 4.5 (Media Cybermetics Inc., Silver Spring, MD, USA). Digitized images were gathered using a Photometrics CoolSNAP$_{fx}$ camera (Photometrics, Surrey, BC, USA) equipped to a Leica DMIRB microscope (Leica Microsystems, Bannockburn, IL, USA). Dimensions of at least 100 cells from 3 to 4 sections per heart were measured. Visual fields were accepted for quantification if nuclei were visible and cell membranes were intact [47, 49].

For electron microscopy, hearts were fixed by perfusion with 2% paraformaldehyde and 2% glutaraldehyde. Tissue from the hearts was then sectioned and stained by the conventional osmium-uranium lead method [15].

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was performed on paraffin-embedded heart slices using DeadEnd™ Fluorometric TUNEL System (Promega, Madison, WI, USA) as per manufacture's instructions [8, 59]. Briefly, tissues were deparaffinized and treated with Proteinase K to permeabilize tissue to the staining reagents. Tissue was equilibrated with equilibration buffer and then incubated with Tdt. TUNEL positive nuclei were distinguished from the TUNEL negative nuclei by counterstaining with

4′,6-diamidino-2-phenylindole (DAPI). For negative controls, heart slices were treated similarly but in the absence of the TdT enzyme. For positive controls, heart slices were treated with DNase I to cause DNA fragmentation. TUNEL-positive cells were measured from digitized images using Image ProPlus Version 4.5 (Media Cybermetics Inc., Silver Spring, MD, USA). Digitized images were gathered using a Photometrics CoolSNAP$_{fx}$ camera (Photometrics, Surrey, BC, Canada) equipped to a Leica DMIRB microscope (Leica Microsystems, Bannockburn, IL, USA) with filters set to view the green fluorescence of TUNEL stained cells (520 nm) and blue DAPI stained cells (460 nm). TUNEL-positive cells were counted in five randomly selected fields per slide and three sections per hearts and averaged for statistical analysis. The percentage of nuclei labeled by TUNEL per unit of cells stained with DAPI reflected the apoptotic index. The observer was blinded to the treatment group.

For measurement of caspase-3 activity, freshly collected heart tissue was homogenized at 4°C with 1 mL of 10× PBS, pH 7.4, and centrifuged with 10, 000×g for 20 min at 4°C. 10 μL of the supernatant was mixed with 40 μL of assay buffer containing (mM) [100 NaCl, 50 HEPES, 10 DTT, 1 EDTA, 10% Glycerol, 0.1% CHAPS, pH 7.4]. 50μL caspase-3 substrate I containing 200 μM of Ac-DEVD-pNA (Alexis Biochemical's, Farmingdale, NY, USA) was added to the mixture. Absorbance was measured at 405 nm [8].

Calcineurin phosphatase activity in 10-week-old heart tissue was assayed using the BIOMOL Green Cellular Calcineurin Assay Kit Plus (BIOMOL Research Laboratories, Plymouth Meeting, PA, USA) [15, 30]. The assay was conducted according to the manufactures instructions. Briefly, tissue was homogenized at 4°C in lysis buffer containing a protease inhibitor and the supernatant was collected. Free phosphates were removed by passing the supernatant through centri-spin 10 columns (Princeton Seperations, Freehold, NJ, USA). To discriminate between the contributions of other phosphatases, total phosphatase activity was measured in the presence of EGTA (to inhibit PP2B), in the presence of 100 nM okadaic acid (to inhibit PP1 and PP2A), and in the presence of EGTA and okadaic acid (to inhibit PP1, PP2A and PP2B, not PP2C). The reaction, carried out in a 96-well microtiter plate, was initiated by the addition of the sample and terminated by the addition of BIOMOL green reagent. The color was allowed to develop for 30 min and optical density was measured at 620 nm. Calcineurin activity was calculated for each group using the following equation, calcineurin activity = total activity in the presence of okadaic acid − (total activity in the presence of okadaic acid and EGTA).

Echocardiography was performed on isoflurane anesthetized mice with A Vevo 770 ultrasound unit (VisualSonics Inc., Toronto, ON, Canada) equipped with a 35 MHz transducer. We used short axis M-mode analysis, at the level of the papillary muscle, for measurement of diastolic and systolic diameters of the left ventricle and wall thickness. Pulsed wave doppler was used at the levels of the left ventricular outflow tract, the right ventricular outflow tract and the mitral valve tips, to monitor flow pattern and velocity. Myocardial performance index or Tei index was calculated using the equation: (isovolumic contraction time + isovolumic relaxation time)/ejection time [18].

Atrial natriuretic peptide (ANP) mRNA expression was measured by real-time PCR as described previously [6, 28]. Heart tissue was homogenized in Trizol reagent according to the manufacturers instruction. The isolated RNA was transcribed to cDNA using Superscript-II. Real-time PCR was performed using SYBR green JumpStart *Taq* ReadyMix DNA polymerase and fluorescence was measured with a DNA Engine Opticon 2 system (MJ Research, Waltham, MA, USA). 18S rRNA was used as a reference gene. Primer sequences and PCR conditions for atrial natriuretic peptide and 18S rRNA were as described previously [28, 61]. Results are presented as the ratio of ANP to 18S rRNA.

Statistical analysis

All values are expressed as mean ± SEM. Groups were compared by both Student t test and Wilcoxon signed-rank test. Differences were considered significant when P values <0.05.

Results

NHE1 protein expression and HA-tagged NHE1 expression

To confirm and quantify NHE1 protein expression in transgenic mice, exogenous and endogenous (total) NHE1 protein expression was measured by western blotting with antibody against the NHE1 protein in control, N-line (wild-type NHE1) and K-line (active NHE1) heart lysates. Figure 1a illustrates that all three samples contained NHE1 protein with the relative amount increasing in N-line and in K-line versus control. Quantification of a series of experiments (Fig. 1a bottom panel) shows that there was a marked increase (two to fourfold) in total NHE1 protein expression in N-line and K-line hearts versus controls, respectively ($P < 0.05$) but no significant differences between K-line versus N-line hearts.

Levels of the exogenous NHE1 protein were also measured by immunoblotting for the HA-tag (Fig. 1b).

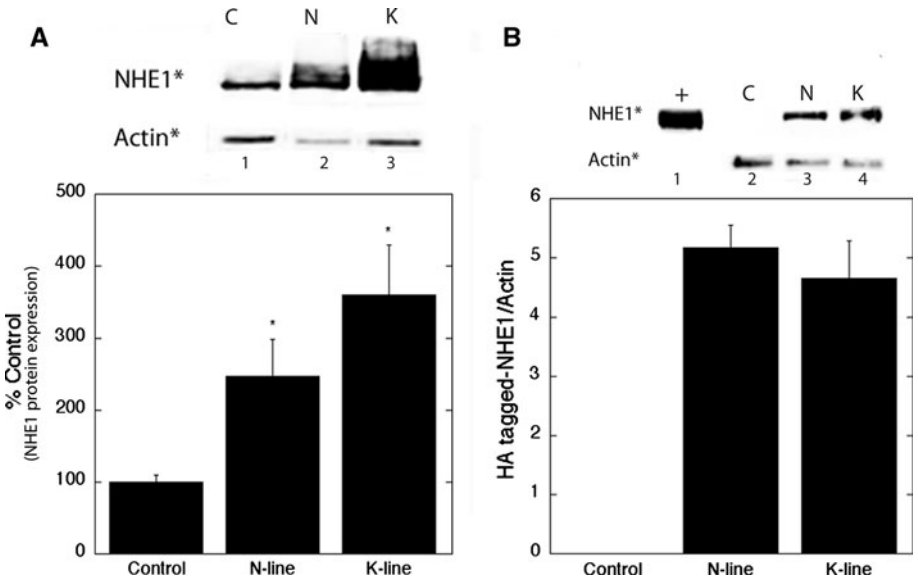

Fig. 1 Immunoblot analysis of NHE1 protein expression. **a** Immunoblot analysis of total exogenous and endogenous NHE1 expression detected by anti-NHE1 antibody. *Upper panel* representative western blot of total NHE1 protein* at 90–100 kDa and of actin * at 40 kDa. *Lanes 1*, *2* and *3* represent heart lysates from control (C), N-line (N) and K-line (K) mice, respectively. *Lower panel* quantification of a series of experiments ($n = 3$–4 hearts/group). NHE1 protein levels were normalized to actin and are expressed as a % of controls ± %SE. *$P < 0.05$ for control versus N-line or K-line. **b** Immunoblot analysis of exogenous HA tagged-NHE1 protein expression. *Upper panel* representative western blot of 90–100 kDa NHE1 and 40 kDa actin. *Lane 1* positive control from CHO cells that were transfected with an HA tagged-NHE1; *lanes 2*, *3* and *4* represent heart extracts of control, N-line and K-line mice, respectively. *Lower panel* NHE1 protein levels were quantified and normalized to actin for each group ($n = 3$–4 hearts/group)

Exogenous NHE1 protein was present in the N-line and K-line hearts, but not in the controls. Chinese hamster ovary cells transfected with HA-tagged NHE1 served as a positive control. Quantification of a series of experiments (Fig. 1b bottom panel) shows no significant difference in exogenous NHE1 protein expression between N-line and K-line hearts.

In vivo indices of cardiac hypertrophy

We examined transgenic mice that had elevated expression of exogenous NHE1 protein. In this study mice were 10–12 weeks of age for use. At this age, there was only a small increase in mortality in K-line mice compared to controls. However, over longer periods of time mortality in the K-line mice was greatly elevated. After 8–9 months, mortality was approximately 60% in K-line mice but was only approximately 20% in N-line mice. At 10–12 weeks of age, there were no apparent changes in grooming or other behavior. Preliminary evidence found that young K-line mice performed poorly in treadmill exercise tests relative to N-line mice and controls (not shown).

To evaluate the effect of NHE1 protein expression various hypertrophic indices were measured, including HW/BW, ANP mRNA expression, CSA and IF. The HW/BW was significantly increased in the K-line mice (0.0072 ± 0.00046 mg/g) versus controls (0.0050 ± 0.00023 mg/g) ($P < 0.005$). In N-line mice (0.0063 ± 0.00047 mg/g), there was a trend towards increased HW/BW, but this was not significant (Fig. 2a). The increase HW/BW in K-line mice was a result of changes in the heart weight (100.0 ± 4.4% controls vs. 126.0 ± 12.3% N-line vs. 157.0 ± 10.3% K-line, $P < 0.005$), as there were only small changes in the body weight (100.0 ± 2.9% controls vs. 98.2 ± 3.8% N-line vs. 109.6 ± 2.1% K-line) and K-line mice increased slightly in body weight. This change in heart weight was due to an increase in heart mass, not edema as indicated by the dry weight to wet weight ratio, which was not significantly different between the groups (0.15 ± 0.014 control and 0.14 ± 0.009 K-line). Another index of cardiac hypertrophy is ANP mRNA expression. Hearts from N-line (0.51 ± 0.16 ANP/18S rRNA) and K-line (0.86 ± 0.15 ANP/18S rRNA) mice had significantly greater ANP mRNA expression in comparison to control (0.041 ± 0.018) mice ($P < 0.05$ for control versus N-line or K-line, Fig. 2b).

Figure 2c–e illustrates the H&E and PSR stained mouse hearts from controls and NHE1 transgenic mice. The CSA measured using H&E heart cross sections was significantly increased in K-line hearts (38,648 ± 4722 arbitrary units), compared to controls (23,566 ± 3535 arbitrary units) or N-line (24,321 ± 364 arbitrary units) hearts ($P < 0.05$). Similarly, IF measured from hearts stained with PSR were also only significantly increased in K-line hearts

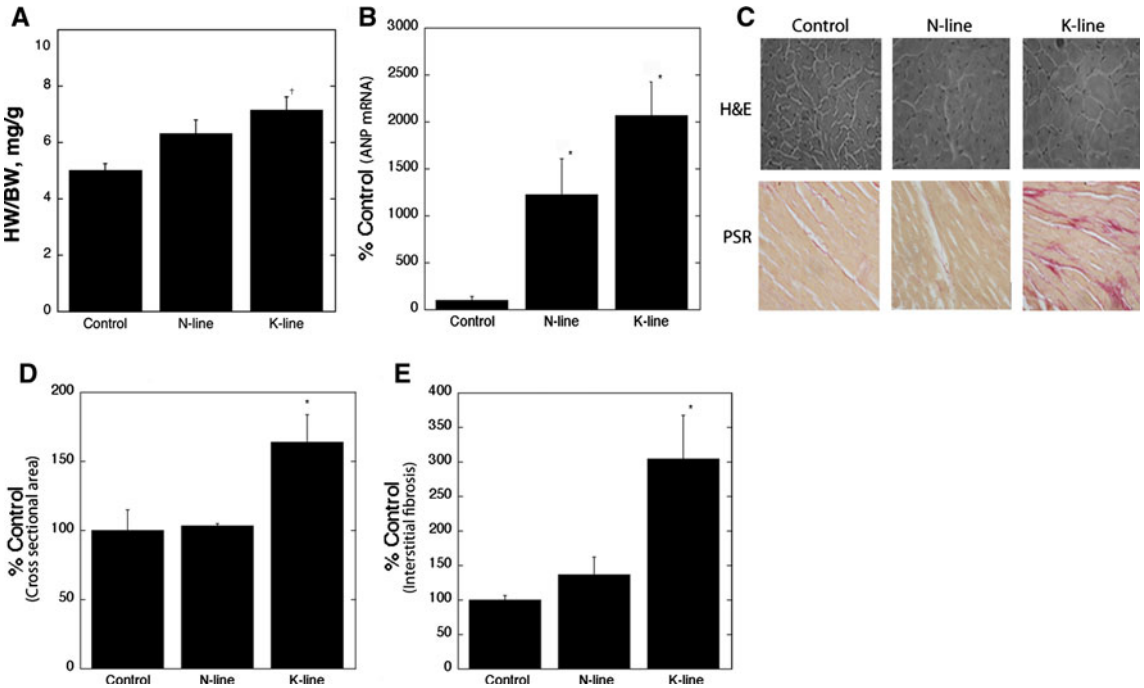

Fig. 2 Analysis of indices of cardiac hypertrophy in N- or K-line mouse hearts. **a** HW/BW of control, N-line and K-line mice. ($^{\dagger}P < 0.005$ for controls vs. K-line, $n = 7$–9 hearts/group). **b** ANP mRNA levels of control, N- and K-line mice. ($n = 3$ hearts/group, $^{*}P < 0.05$ for control vs. N- or K-line. **c** *Upper panel* representative heart cross sections (×40) of control, N- and K-line hearts stained with H & E. *Lower panel* heart sections stained with picro-sirius red. **d** Quantitative analysis of CSA ($^{*}P < 0.05$ for K-line vs. control, $n = 4$–6 hearts/group). Results are expressed as a % of controls ± %SEM. **e** Quantitative analysis of IF. ($^{*}P < 0.05$ for controls vs. K-line, $n = 4$ hearts/group, results are expressed as a % of controls ± %SEM)

(0.050 ± 0.01% stained) compared to controls (0.017 ± 0.001% stained) or N-line hearts (0.023 ± 0.004% stained) ($P < 0.05$).

Cardiac function and myocardial remodeling in vivo

Echocardiographic measurements of cardiac function and morphology of mice are summarized in Table 1. Compared to control (or N-line) mice, hearts from K-line mice exhibited significant ventricular hypertrophy. Left ventricular mass (LVM) of K-line mice was significantly elevated as were diastolic inter-ventricular septal wall thickness (IVSTd) and diastolic left ventricular posterior wall thickness (LVPWd). These parameters were not significantly elevated in N-line mice. Heart rate was also not significantly different between control, N-line and K-line mice.

Both diastolic and systolic cardiac functions were significantly affected, principally in the K-line mice and to a lesser degree in the N-line mice. In N-line and K-line mice, the decrease in systolic function was demonstrated by a decrease in percent left ventricular ejection fraction (EV). The decrease in diastolic function was demonstrated by the increase in the ratio of peak E wave mitral valve velocity to peak A wave mitral valve velocity (E/A, ratio). This decrease in systolic and diastolic function in N-line and K-line mice is associated with ventricular dilation, as indicated by the increase in left ventricular internal dimension (LVID) during systole—more so in K-line mice

Table 1 Cardiac morphology and contractile function of control, N-line and K-line mice

$n = 7$–8/group	Control	N-line	K-line
Heart rate, bpm	406 ± 58	407 ± 36	414 ± 54
IVSTd, mm	0.71 ± 0.04	0.77 ± 0.06	0.83 ± 0.1*
LVPWd, mm	0.74 ± 0.05	0.74 ± 0.07	0.84 ± 0.04*, §
LVM, mg	81.3 ± 5.9	85.9 ± 8.4	103.4 ± 13.1†, §
LVIDd, mm	3.98 ± 0.24	3.98 ± 0.16	4.10 ± 0.23
LVIDs, mm	2.44 ± 0.16	2.69 ± 0.21*	3.12 ± 0.24*, §
FS, %	38.8 ± 2.0	32.4 ± 3.0†	24.0 ± 3.5†, ¥
EF, %	69.8 ± 2.4	61.2 ± 4.4†	48.1 ± 5.8†, ¥
E/A ratio	1.95 ± 0.3	2.65 ± 0.54*	4.04 ± 1.6*
Tei index	0.457 ± 0.06	0.5 1 ± 0.04	0.70 ± 0.09†, ¥

Values are expressed as mean ± SD. $^{*}P < 0.05$ versus control, $^{\dagger}P < 0.005$ versus control, $^{\S}P < 0.05$ for N-line versus K-line, $^{¥}P < 0.005$ for N-line versus K-line

IVSTd diastolic inter-ventricular septal wall thickness, *LVPWd* diastolic left ventricular posterior wall thickness, *LVM* left ventricular mass, *LVIDd and LVIDs* left ventricular internal dimension during diastole and systole, *FS* left ventricular % fractional shortening, *EF* left ventricular % ejection fraction, *E/A ratio* ratio of peak E wave mitral valve velocity to peak A wave mitral valve velocity

($P < 0.05$ vs. control). The global deterioration in myocardial performance is evident only in K-line mice as indicated by the significant increase in the Tei index ($P < 0.005$). In addition, changes in Tei index, left ventricular fractional shortening (FS) and EF were significantly greater in K-line mice compared to N-line.

Signaling pathways of transgenic hearts

We examined expression and activation of signaling pathways in vivo to determine if they were involved in NHE1-mediated remodeling of the heart. We examined ERK, JNK, p38 and RSK. The level of phospho-ERK, phospho-p38, p38α, phospho-JNK or phospho RSK was not elevated in 12-week-old heart lysates from N-line and K-line mice when compared to controls (Suppl. Fig. 1A). To determine if signaling pathways were activated at a younger age we also examined phospho-ERK, phospho-p38, and phospho RSK 2-week-old hearts from transgenic mice. There were no significant increases in the level of phospho-ERK, phospho-p38, phospho-JNK or phospho RSK in these mouse hearts (Suppl. Fig. 1B).

We also examined GSK-3β protein expression in both the cytosolic and mitochondrial fractions. There were no significant changes in the GSK-3β protein levels (Suppl. Fig. 1C).

Finally, we examined the calcineurin activity. We found that there was no difference in calcineurin activity in heart lysates from control, N-line and K-line mice (Suppl. Fig. 1D).

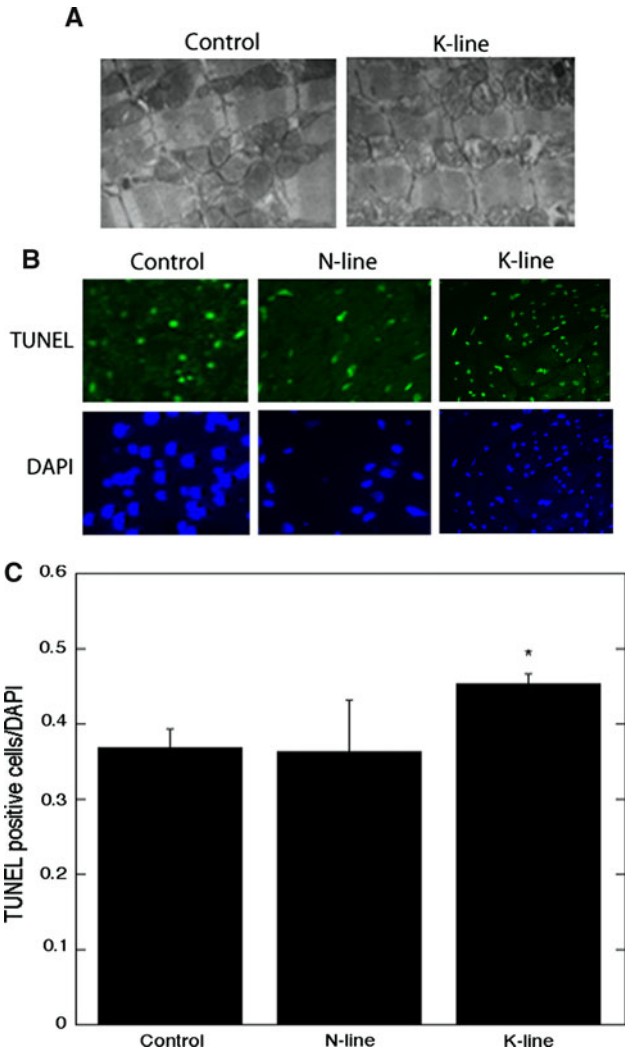

Fig. 3 Examination of left ventricles of control and transgenic mice. **a** Electron micrographs of control and K-line left ventricles. **b** Representative heart cross sections of control, N- and K-line stained with TUNEL (*top*) and DAPI (*bottom*). **c** Quantification of experiments measuring TUNEL straining per DAPI staining in control, N- and K-line ventricles. (Results are expressed as a ratio of TUNEL/DAPI ± SEM, *$P < 0.05$ for control vs. K-line, $n = 3$–4 hearts/group)

Indices of apoptosis in vivo

We examined apoptosis in N-line and K-line hearts. Initially, we examined left ventricular sections from control and K-line hearts using transmission electron microscopy. The mitochondria appeared swollen in K-line versus control hearts (Fig. 3a). We then measured the levels of apoptosis in sections of these hearts by TUNEL staining. K-line hearts showed a significant increase in TUNEL-stained positive cells (123 ± 3.8% vs. 100 ± 6.8% controls; $P < 0.05$) (Fig. 3b, c).

To further understand the mechanisms behind apoptosis in K-line hearts, we examined markers of both the caspase-dependent and independent pathways. For the caspase-dependent pathway, we examined caspase-3 activity in the cytosolic fraction and Cyt c in the mitochondrial and cytosolic fraction. Caspase-3 activity in the cytosolic fraction of heart lysates was not significantly different in K-line versus control hearts (Suppl. Fig. 2A). Immunoblot analysis examining Cyt c protein levels in mitochondrial and cytosolic fractions of control and transgenic hearts (Suppl Fig. 2B) showed there were no changes in the level of Cyt c in control versus transgenic hearts. We also determined the extent of PARP cleavage by caspase-3 (Suppl. Fig. 2C). Control, N-line and K-line hearts had comparable levels of cleaved PARP (85 kDa).

In addition, to investigate the contribution of caspase-independent pathways to apoptosis, AIF protein expression was measured in the mitochondrial and cytosolic fractions of control and transgenic hearts (Fig. 4). Immunoblot analysis showed that AIF protein expression (normalized to MnSOD) was significantly greater in the mitochondrial fraction of K-line hearts (165 ± 13.6 vs. 100 ± 16.3 control; $P < 0.05$). In the cytosolic fraction, control, N-line

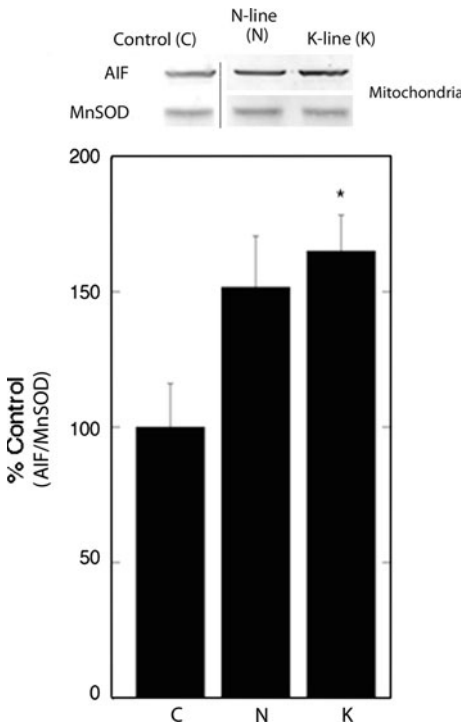

Fig. 4 Analysis of apoptosis mediated through the caspase independent mitochondrial pathway in control, N-line and K-line mice. *C* control, *N* N-line and *K* K-line. *Upper panel* representative western blotting for AIF and MnSOD protein expression in the mitochondrial fraction from heart lysates of control, N- and K-line mice. *Lower panel* quantification of a series of experiments normalized for each sample to the internal control (Results are expressed as a % of controls ± %SEM, $*P < 0.05$ for controls vs. K-line, $n = 6$ hearts/group)

and K-line hearts all had much weaker expression of AIF (normalized to GAPDH) but was unchanged between the different mouse lines (not shown).

Alpha$_1$-adrenergic stimulation in vivo

Increased sympathetic activity has been implicated in the development of cardiac hypertrophy in animal models [21, 33] and in humans [26]. One well used model of cardiac hypertrophy is administration of PE to intact animals [5, 44, 54, 60]. To test the susceptibility of control, N-line and K-line mice we used this model of PE-mediated hypertrophy. PE was administered by implanted Alzet osmotic minipumps for a period of 2 weeks. K-line mice stimulated with PE (0.0095 ± 0.00079 mg/g) had a significantly increased HW/BW versus K-line mice treated with vehicle (0.0072 ± 0.00046 mg/g) (Fig. 5a, $P < 0.05$). Hearts from control mice stimulated with PE (0.0056 ± 0.00041 mg/g) also showed a trend towards increase in the HW/BW, although this was not significant with this low dose of PE. Hearts from N-line mice stimulated with PE (0.0063 ± 0.00047 mg/g) showed no difference versus hearts from N-line mice treated with PBS (0.0062 ± 0.000191 mg/g). The variations seen in the HW/BW with PE treatment were the result of changes in the heart weight, as there were no significant changes in the body weight (data not shown). However, this change in heart weight in the K-line mice was, at least in part, due to edema. When we examined the dry weight to wet weight ratio of hearts from K-line mice stimulated with PE (0.14 ± 0.0086), we found it had decreased versus hearts from K-line mice treated with PBS (0.096 ± 0.013) (Fig. 5b, $P < 0.05$). There was no change in the dry weight to wet weight ratio of hearts from control mice treated with PE (0.16 ± 0.015) versus hearts from control mice treated with PBS (0.15 ± 0.014).

We also examined ANP mRNA expression; comparing the ANP expression of hearts from PE-treated mice with

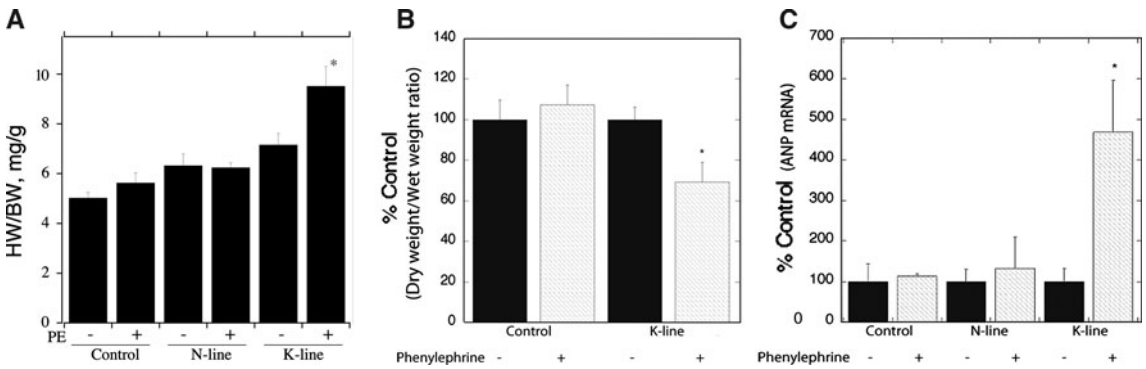

Fig. 5 Analysis of indices of cardiac hypertrophy in control, N- and K-line mice stimulated with PE. **a** HW/BW, (stimulated with either vehicle, PBS (−) or PE (+), ±% SEM. $*P < 0.05$ for K-line stimulated with PBS versus K-line stimulated with PE ($n = 7$–11 hearts/group). **b** Dry tissue weight/wet tissue weight, in control or K-line hearts in the presence or absence of PE or PBS. Results are expressed as % of control (stimulated with vehicle, PBS) ± % SEM. ($*P < 0.05$ for K-line stimulated with PBS vs. K-line stimulated with PE, $n = 3$–4 hearts/group). **c** ANP mRNA levels determined by real-time PCR. Results are the ratio of ANP to 18S rRNA and expressed as % of each individual control group stimulated with vehicle, PBS. ($*P < 0.05$ for K-line stimulated with PBS vs. K-line stimulated with PE, $n = 3$ hearts/group)

controls (those treated with PBS). As shown in Fig. 5c, ANP mRNA expression was only elevated in hearts from K-line mice stimulated with PE (4.18 ± 2.2 ANP/18S rRNA) versus hearts from K-line mice stimulated with PBS (0.86 ± 0.28 ANP/18S rRNA) ($P < 0.05$). Stimulation with PE had no significant effects on CSA and IF in control, N-line and K-line mice when comparing PBS treated with PE treated (Suppl. Fig. 3A, B).

Endoplasmic reticulum stress response

Previous reports have suggested that the ER stress response is likely to have contributed to a cardioprotective effect that is observed in transgenic mice expressing elevated NHE1 following ischemia/reperfusion-injury [13]. Therefore, we examined the level of calreticulin and PDI, proteins that are involved in the ER stress response. In agreement with previous reports, heart extracts from N-line mice had significantly greater levels of calreticulin and PDI protein expression versus control ($P < 0.005$, Fig. 6). Heart extracts from K-line mice also had significantly greater calreticulin and PDI protein expression versus control ($P < 0.005$, Fig. 6). PDI protein expression in K-line heart extracts was also significantly greater than PDI protein expression in heart extracts from N-line mice ($P < 0.05$) (Fig. 6).

Discussion

We examined the effects of elevated expression of a wild type and activated NHE1 on the myocardium in vivo in intact mice. We have previously used this established mouse model and shown that elevation of NHE1 makes the myocardium less susceptible to ischemia/reperfusion-induced injury [34], an observation later confirmed by others [13]. The long-term effects of expression of an activated from of NHE1 compared with the wild type, have not been examined. This is a physiologically relevant question as increased NHE1 expression and activity are reported in several in vivo and in vitro models of cardiac diseases [19–21, 27, 38, 62]. NHE1 also has a set point and is inactive at pH's near 7.4 [14]. Therefore, it is uncertain whether increased elevation of NHE1 expression alone has detrimental effects without the activation that shifts it to greater activity at more alkaline pH. Such activation could occur in pathological situations. For example, kinases that phosphorylate NHE1 have increased activity in the diseased myocardium [51]. However, aside from its pH regulatory role NHE1 also has other physiological roles in the cells such as acting as an anchor for other proteins including the actin cytoskeleton [50]. Increased NHE1 expression without increased activity could, therefore, affect cardiac function. We, therefore, investigated the relative contribution of activated versus non-activated NHE1 protein in two different cardiac models.

NHE1 requires enhanced activity to lead to rapid remodeling and myocardial dysfunction

NHE1 generated a pro-hypertrophic effect but this effect was principally in K-line mice expressing an activated form

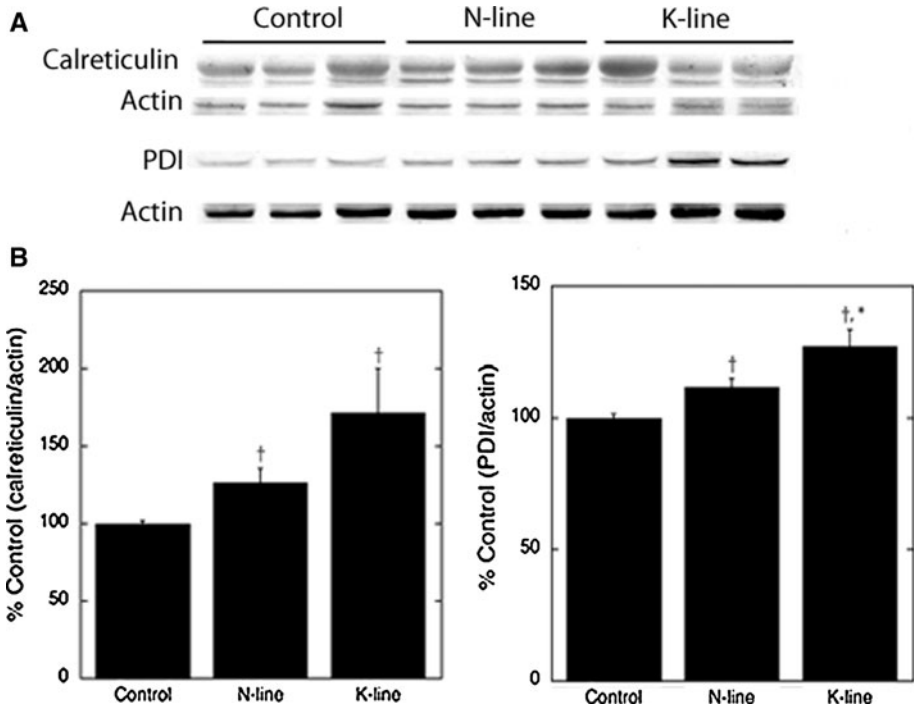

Fig. 6 Immunoblot analysis of endoplasmic reticulum stress response protein expression levels in control, N-line and K-line mouse heart lysates. **a** Western blot of calreticulin and PDI protein expression normalized to actin. **b** Quantification of a series of experiments measuring the ratio of calreticulin or PDI to actin. Results are expressed as a % of control ± SEM. ($n = 5$-6 hearts/group). $^{\dagger}P < 0.005$ for control versus N- or K-line. $^{*}P < 0.05$ for N-line versus K-line

of NHE1. K-line mice demonstrated increased HW/BW, elevated ANP expression, elevated IF and increased CSA. N-line mice only showed elevated ANP mRNA levels. Echocardiography revealed that K-line mice had a number of morphological defects and global myocardial deterioration while N-line mice were similar to wild type, with mild functional abnormalities. The results with the K-line mice are largely in agreement with a recent study [52] that also expressed activated NHE1 in the myocardium. However, their study used a large deletion of the NHE1 C-terminus (637–656 base pair region), as opposed to the specific point mutations in the calmodulin binding region used in the present study. In addition, we examined 10–12-week-old mice as opposed to 40 day-old mice. Our findings directly distinguish between the effects of increased expression of (wild type) NHE1 versus increased expression of an activated NHE1, which was not shown in the earlier work [52]. We showed that expression of the activated NHE1 causes strong hypertrophic effects while expression of the wild type NHE1 causes milder effects. Thus, the activated form of NHE1 was required to induce (most) phenotypic changes in the whole animal—expression of wild type NHE1 had milder effects. Cook et al. [13] demonstrated that enhanced expression of wild type NHE1 results in spontaneous development of heart failure, premature mortality, increased apoptotic cell death and cardiac remodeling in another mouse model. However, their results were for much older mice, 8–11 months of age, as opposed to ours at 10–12 weeks. This supports the idea that expression of wild type NHE1 has a weaker pro-hypertrophic effect. Given the long periods of time in older mice, this effect appears to accumulate and causes more severe pathological changes.

Signaling pathways leading to cardiac hypertrophy

Numerous signaling pathways have been implicated in the development of cardiac hypertrophy including MAPKs (ERK, p38 and JNK) and RSK [31]. Many of these pro-hypertrophic signaling pathways have also been implicated in regulation of NHE1 [3, 46]. We found no significant differences in phosphorylated ERK, p38, JNK, RSK in our N-line and K-line mice when compared to control. In contrast, a previous report [52] in which active NHE1 was over-expressed, found that p38 and ERK were significantly activated. Our model varied from that reported earlier in the region mutated on the C-terminus of NHE1. We used site-specific mutagenesis to cause a specific mutation of the calmodulin regulatory domain. The earlier model used deletion of amino acids 637–656. This appears to have caused a more extreme activation of the NHE1 protein, inducing further stress and perhaps contributing to the activation of MAPKs. The early and more severe effects of NHE1 expression shown in that study [52] support this suggestion. If activation of these kinases only occurs with later severe stages of the pathology, the activation of these pathways is likely a later consequence of the heart failure, rather than being causal. An alternative explanation for the discrepancy between the studies is that that these kinases were activated at a different age in our model of transgenic animals. We examined two time points, 2 and 12-week-old hearts. It is possible that activation of the kinases occurred at ages other than these time points. Future studies will examine this possibility.

We also did not find any differences in total GSK-3β and calcineurin activity in N-line and K-line mice. This suggested that these pathways are also not involved in mediating the pathology that we observed. It has been suggested that alteration of Ca^{2+} cycling [52] mediates the cardiac hypertrophy induced by expression of activated NHE1 in the myocardium. We previously demonstrated that N-line and K-line mice do not have an alteration of expression of genes involved in Ca^{2+} cycling (NCX, SERCA and Na^{+}-K^{+}-ATPase) and pH regulatory proteins (AE3 and NBC1) [34]. Preliminary results have also suggested that mRNA levels of these Ca^{2+} cycling proteins are unaltered in the transgenic mice (unpublished observations). However, we have not excluded alteration of Ca^{2+} handling in these mice and in fact, we noted earlier that K-line mice have abnormal calcium handling [4], which could be involved in mediating cardiac hypertrophy.

ER stress response protein levels

We also investigated the involvement of the ER stress response, which was previously suggested to contribute to the cardioprotective effects of elevated expression of NHE1 [13]. Induction of multiple ER stress associated genes has been reported to lead to a dramatic reduction in infarct size following I/R-injury [48]. In our studies, we found that both N-line and K-line mouse hearts expressed elevated levels of the ER stress proteins calreticulin and PDI. K-line mouse hearts expressed more stress response protein than N-line mice and this difference was significant for the level of PDI versus N-line hearts. The long-term elevation of ER stress proteins leads to cardiac remodeling and failure [13] which may account for some of the effects we found including K-line mice hypertrophy and sensitivity to alpha$_1$-adrenergic stimulation (see below).

Apoptosis and NHE1

Apoptosis can be initiated by the death receptor/extrinsic pathway, the mitochondrial/intrinsic pathway [29] and unresolved ER stress [13]. In previous reports, it was demonstrated that chronic elevation of NHE1 expression produces elevation of both ER stress proteins and the

expression of the C/EBP homologous protein, which mediates the induction of pro-apoptotic pathways [13]. The elevation of ER stress proteins was initially protective to the myocardium, but later, there was a biphasic effect and chronic ER stress facilitated cardiac remodeling and failure, likely through induction of apoptosis [13]. However, the mitochondrial/intrinsic pathway was not investigated and ruled out as means of inducing apoptosis in NHE1 expressing transgenic mice. This pathway has been implicated in NHE1-mediated apoptosis in the myocardium [29]. In addition, mitochondrial fission and fusion proteins have been shown to be elevated in cardiomyocytes during phenylephrine induced hypertrophy [36]. In our study, we showed swollen mitochondria in our K-line mice. In addition, there was significantly elevated apoptosis in the myocardium. Apoptosis was not demonstrated in hearts expressing wild type NHE1 indicated that an activated NHE1 was facilitating this effect. Cook et al. [13] found that wild type NHE1 induces apoptosis, however, they examined much older mice, and the effects of elevated NHE1 expression increased with age. The effects of expression of NHE1 with age were also clearly demonstrated in our earlier studies, which examined the survival rate of N-line and K-line mice. The survival rate of N-line mice declined only slightly following 7 months of age, while only 40% of the K-line mice were alive at 8 months of age [12]. Further studies characterizing the age-induced phenotype of transgenic mice expressing active NHE1 would be essential to providing further understanding of the role of NHE1 in cardiac pathology.

We further investigate the contribution of the caspase dependent pathway to apoptosis. We observed no differences in Cyt c and cleaved PARP protein expression in both the cytosol and mitochondria in control and NHE1 expressing transgenic mice. AIF has been shown to translocate into the cytoplasm with further induction into the nuclei and this induces a caspase-independent DNA fragmentation [55]. Our results demonstrate that AIF appears to be significantly greater in the mitochondrial fraction of our K-line mice. Since release of AIF from mitochondria is normally thought to trigger the cell death program, our results suggest that cell death is likely not mediated through this mitochondrial pathway. Therefore, although it is evident that activated NHE1 induces apoptosis, the mechanism by which this occurs seems less likely to involve the mitochondrial/intrinsic pathway, supporting the suggestion that ER stress is involved [13].

NHE1 expression sensitizes the myocardium to alpha$_1$-adrenergic stimulation

We noticed that K-line mice appeared to be particularly sensitive to the stress induced by pregnancy, causing a relatively high rate of mortality during gestation that was associated with cardiovascular failure (not shown). We, therefore, examined whether these mice were more sensitive to the cardiovascular stress of alpha$_1$-adrenergic stimulation, which both causes cardiac hypertrophy and stimulates NHE1 activity [11, 41, 43, 56, 63]. In heart failure, chronic stimulation of adrenoceptors has been linked to pathological cardiac remodeling, including cardiomyocyte apoptosis and hypertrophy [57]. In addition, beneficial effects of NHE1 inhibitor treatment in a rapid pacing model of heart failure may be due to attenuation of increases in circulating noradrenalin content [42]. We found that stimulation of K-line mice with PE led to a further increase in HW/BW and a further increase in ANP mRNA expression. The effect of PE on K-line mice was associated with myocardial edema, possibly indicating a later stage of chronic heart failure. The large elevation of ANP mRNA levels by PE in the K-line mice indicates that there was a further progression of the hypertrophic response. Other indicators such as CSA and IF were not further elevated, perhaps due to the degree of NHE1-induced hypertrophy. The results suggest that the presence of the activated NHE1 causes an increase in the sensitivity to PE stimulation particularly in vivo in mice.

Overall, our data demonstrate that activated NHE1 is necessary for the more severe pathological effects of NHE1 expression in vivo. Elevated expression of wild type NHE1 only caused relatively minor increases in indices of cardiac hypertrophy. With time, these may cause more detrimental effects. Our model did not show increases in ERK, p38, JNK, RSK phosphorylation or elevated calcineurin levels, suggesting that these are not causal in these stages of the pathology examined. Rather they may be manifestation of later stages of the pathology. An additional novel finding was that expression of activated NHE1 protein causes increased sensitivity to neurohormonal stimuli in intact mice. This is in line with the idea that activation of the increased levels of NHE1 are required to stimulate the cardiac pathology that NHE1 induces. Though NHE1 has other roles in cells such as kinase scaffolding and cytoskeletal anchoring [50], it appears that elevated NHE1 activity is the critical factor in mediating the cardiac pathology.

Acknowledgments This work was supported by the Canadian Institutes of Health Research #MOP-97816. Larry Fliegel is supported by an Alberta Heritage Foundation for Medical Research Senior Scientist award and Fatima Mraiche is supported by CIHR and AHFMR. Morris Karmazyn is supported by a Canada Research Chair in Experimental Cardiology.

Conflict of interest None.

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