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Expression and characterization of the Na^+/H^+ exchanger in the mammalian myocardium

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Abstract We examined two expression systems for studying the Na^+/H^+ exchanger in the mammalian myocardium. Mammalian NHE1 with a hemagglutinin (HA) tag and was cloned behind the alpha myosin heavy chain promoter. Transgenic mice were made with wild type NHE1 protein or with a hyperactive NHE1 protein mutated at the calmodulin-binding domain. Three lines of transgenic mice were made of each cDNA with expression levels of each type varying from high to low. Higher levels and activity of the Na^+/H^+ exchanger were associated with decreased long-term survival of mice, and with dilated or hypertrophic cardiomyopathy. The exogenous NHE1 protein was present in freshly made cardiomyocytes from transgenic mice, however, expression from the alpha myosin heavy chain promoter declined rapidly and little exogenous NHE1 was apparent on the fourth day after cardiomyocyte isolation. To express NHE1 protein in isolated cardiomyocytes, we transferred a mutated form of the protein into an adenoviral expression system. Infection of neonatal rat cardiomyocytes resulted in robust expression of the exogenous NHE1 protein. The mutant form of the NHE1 protein could be distinguished from the endogenous Na^+/H^+ exchanger by its resistance to inhibition by amiloride analogs. Our results suggest that for in vivo studies on intact hearts and animals, expression in transgenic mice is an appropriate system, however for long-term studies on cardiomyocytes, this model is inappropriate due to waning expression from the alpha myosin heavy chain promoter. Therefore, infection by adenovirus is a superior system for long-term studies on cardiomyocytes in culture.

Keywords Sodium/hydrogen exchange · Intracellular pH · Heart hypertrophy · Adenovirus

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Introduction

The Na^+/H^+ exchanger (NHE) is a ubiquitous plasma membrane glycoprotein that plays a key role in intracellular pH regulation. Within the plasma membrane of cells it extrudes one intracellular proton in exchange for one extracellular sodium, removing excess intracellular acid that is a product of metabolism [1]. Of the nine isoforms described so far the NHE1 isoform (NHE1) is the only plasma membrane isoform present in cardiac tissue [2]. NHE1 is extremely important in the myocardium it has been implicated in myocardial ischemia/reperfusion (I/R) injury [2] and in cardiac hypertrophy following myocardial infarction [3]. During myocardial I/R, intracellular pH decreases due to metabolic demands that lead to proton production. This excess intracellular acid that is produced during ischemia leads to increased NHE1 activity and results in elevated intracellular sodium, $[Na^+]_i$. The increased $[Na^+]_i$ promotes reverse-mode Na^+/Ca^{2+} exchanger activity leading to Ca^{2+} overload and Ca^{2+} overload can lead to contractile dysfunction, cell death or arrhythmias [4–6]. A large number of studies have shown that inhibition of the Na^+/H^+ exchanger during I/R prevents myocardial injury [7–11].

In addition to its critical role in I/R injury, studies have suggested that NHE1 activity and expression are critical in heart hypertrophy [2, 12, 13]. We [14] and others [14–17] have shown that NHE inhibition can prevent the remodeling

of the myocardium induced by hypertrophic stimuli. These data suggest that increased NHE1 expression plays a critical role in the detrimental effects on the myocardium. It has also been shown that NHE1 expression can be increased in the myocardium in response to ischemia and acidosis [18, 19].

Since the Na^+/H^+ exchanger plays a critical role in various forms of heart disease, and expression and regulation of the protein varies in the pathological setting [20] there is an inherent interest in studying the effects of elevated levels of NHE1 protein in the myocardium. Therefore, we compared two different expression systems for their efficacy in studying expression of exogenous NHE1 protein in the heart. Cardiomyocytes are quite refractory to lipid or calcium based transfection systems. We therefore examined a transgenic model of NHE1 expression in the mouse myocardium, and examined its usefulness in studying isolated cardiomyocytes from the myocardium of these animals. We also compared this with the adenovirus system of transfection of cardiomyocytes. Surprisingly, we found that while freshly made cardiomyocytes from transgenic mice contained exogenous Na^+/H^+ exchanger protein, expression from the alpha myosin heavy chain promoter declined rapidly in culture. These results suggest that transgenic mice make a suitable model for expression in intact animals, in isolated perfused hearts and in freshly prepared cardiomyocytes. However, for cardiomyocytes kept in culture, adenoviral expression is more suitable for long-term experiments.

Materials and methods

Materials- Collagenase Type 2 was obtained from Worthington Biochemical Corporation (Lakewood, NJ) and BCECF-AM was from Molecular Probes (Eugene, OR). Platinum R Taq Polymerase was from In Vitrogen (Burlington, Ont.). All other chemicals were of analytical grade and were purchased from Fisher Scientific (Ottawa, ON), Sigma (St. Louis, MO) or BDH (Toronto, ON). EMD87580 was a generous gift of Dr. N. Beier of Merck KGaA, Frankfurter, Germany. HOE 694 was from Aventus (Frankfurt, Germany). PWO DNA polymerase was from Roche Applied Science and Lipofectamine^{TM} 2000 reagent was from Invitrogen.

Construction of Na^+/H^+ exchanger transgenic mice

We created two types of transgenic mouse lines, the N and K-lines. The N-line expressed the wild type Na^+/H^+ exchanger isoform 1 (NHE1) in the mouse myocardium. Expression of the hemagglutinin (HA) tagged Na^+/H^+ exchanger [21] was targeted to the myocardium via the alpha myosin heavy chain promoter. The plasmid pYN4+ contains the cDNA of the entire coding region of the Na^+/H^+ exchanger [22]. A *Sma*I and *Nde*I digest of the pYN4+ plasmid yielded a 2551 bp piece that contained the full length cDNA of the NHE1 isoform of the Na^+/H^+ exchanger with an HA tag at its C-terminus. The *Nde*I site was blunted and the fragment was cloned into a plasmid containing the 5.5-kb mouse cardiac alpha myosin heavy chain promoter. It was cloned into a *Sal*I site immediately downstream of the promoter that was filled in with Klenow enzyme. DNA in the correct orientation linear was excised from the plasmid using the enzyme *Not*I. The linear fragment of alpha-MHC-NHE1-HA (Fig. 1A) was microinjected into fertilized oocytes that were transferred into the oviduct of pseudopregnant FVB/N mice. PCR analysis of ear genomic DNA was used to identify transgenic mice. A forward primer corresponded to the 3′ end of the alpha-MHC promoter sequence (MHCNHEf 5′-GCCCAGCT GCCCGGCACTCTTAG-3′) and a reverse primer corresponded to the 5′ end of the NHE1 cDNA sequence (MHCNHEr 5′-GCCCCACCAAAGCAACCACCAC-3′). Founder mice were bred with wild-type FVB/N in a pathogen free environment.

The K-line was made with a mutation in the cytoplasmic regulatory calmodulin-binding domain of the protein. The mutation of amino acids Lys641, Arg643, Arg645 and Arg647 to Glu residues (1K3R4E) causes an alkaline shift in NHE1 pH dependency [23]. Site specific mutagenesis used forward and reverse synthetic oligonucleotides of the sequence, 5′-CGCAAAATCCTGAG GAACAACTTGCAGGAGACCGAGCAGGAGCTC-GAGTCCTACAACAGACACACGCTGG-3′) and introduced overlapping *Sac*I and *Xho*I sites to facilitate mutant identification.

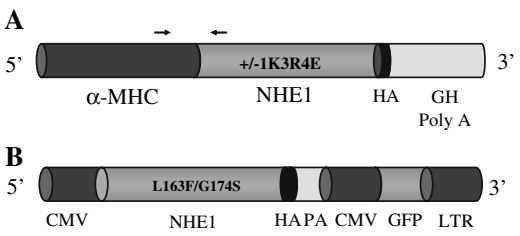

Fig. 1 Na^+/H^+ exchanger gene constructs used for expression of the NHE1 protein in the myocardium. (**A**) Transgene construct for cardiac specific expression of NHE1 in transgenic mice. Arrows indicate the position of primers used to confirm NHE1 presence in transgenic mice (**B**) Adenovirus construct used for expression of NHE1 in isolated cardiomyocytes. αMHC, alpha myosin heavy chain promoter; CMV, cytomegalovirus promoter; GFP, green fluorescent protein; GH, growth hormone poly adenylation signal; HA, hemagglutinin tag; LTR, long terminal repeat; ±1K3R4E indicates that DNA may have contained the mutation of lys641, Arg643, Arg645 and Arg647 to Glu residues

M-mode echocardiography

Transgenic and control mice were anesthetized with methoxyflurane and maintained at 37°C to prevent hypothermia. For cardiac imaging, we used a Philips 5500 cardiac ultrasound system with a 12-MHz phase array sector transducer. The system permits two-dimensional echocardiographic studies using both pulsed and color Doppler intra- and extracardiac blood flow interrogation. M-mode images were obtained in the parasternal short and long views at the papillary muscle level.

Morphometric analysis

Hearts were fixed in 10% neutral buffered formalin. Fixed hearts were embedded in paraffin and sections were stained with Hematoxylin and Eosin.

Construction of Adenoviral NHE1 Gene

The NHE1 gene was used to make an adenoviral gene for transfection of primary cultures of neonatal rat cardiomyocytes. Initially the HA tagged Na^+/H^+ exchanger [21] was mutated to make it resistant to inhibition by amiloride analogues. The NHE1 isoform of the exchanger (that is present in isolated myocytes) is sensitive to inhibition by amiloride analogues. Other isoforms of the NHE (i.e. NHE3) are much more resistant to inhibition [24]. The double mutation Leu163Phe/Gly174Ser in NHE1 increases the resistance to inhibition similar to NHE3 resistance. This mutation was made in the NHE1 in the plasmid pYN4+ as described earlier [21]. The primers for this mutant were pYN4L163Ff GACGTCTTCTTCtTCTTCCTcCTcCCGC CCATCATCC, pYN4L163Fr GGATGATGGGCGGgAGgAGGAAGAaGAAGAAGACGTC and pYN4+Gly174Sf, CATCCTGGATGCtagCTACTTCCTGCCAC pYN4 + -Gly174Sr GTGGCAGGAAGTAGctaGCATCCAGGATG. Mutations (lower case) were also introduced to remove restriction enzyme sites used for screening plasmids.

To construct an adenoviral vector for expression of the NHE1 isoform of the Na^+/H^+ exchanger the mutated, inhibitor resistant Na^+/H^+ exchanger was amplified using a high fidelity polymerase (Platinum R Taq Polymerase, In Vitrogen) from the plasmid pYN4 + using the primers MackpYNF CCGGGGTACCGCCACCATGGTTCTGCG GTCTGG and MackPynR GGAAGCTTAAGCTTC-TACTGAGCAGCGTAATCTGGAAC that flanked the insert with *Kpn*I and *Hin*dIII sites respectively. The product was cloned into an adenovirus using a modified system [25]. The vector pAdTrack-CMV and the PCR product were digested with *Kpn*I and *Hin*dIII. After directional ligation the product was sequenced to confirm identity. The pADTrack-CMV-NHE1 construct was linearized with *Pac*I and used to transform *E. coli* (BJ5183) that contains the vector pAdEasy-1. Recombinant pAd-NHE1 were screened by restriction enzyme mapping, amplified, purified and used to transfect 293A cells (E1-transformed human embryonic kidney cells). Transfection was monitored by GFP expression and for large scale production of adenovirus, viral containing supernatants were used to infect cells. High titer viral stocks were purified by CsCl centrifugation, followed by dialysis and titering using the plaque assay method.

Preparation and characterization of isolated cardiomyocytes

Primary cultures of cardiomyocytes were prepared from neonatal Sprague Dawley rat heart ventricles as described previously [26] or 1-month old transgenic mice. Isolated primary myocytes were plated onto glass coverslips for physiologic studies, or onto Primaria^{TM} (Falcon) culture dishes or flasks. Myocytes were maintained for 4–5 days in medium containing Dulbecco's modified Eagle's medium (DMEM/F12) supplemented with 10% bovine growth serum (FBS), 10 μg/ml transferrin, 10 μg/ml insulin, 10 ng/ml selenium, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mg/ml bovine serum albumin (BSA), 5 μg/ml linoleic acid, 3 mM pyruvic acid, 0.1 mM minimum essential medium (MEM) non-essential amino acids, 10% MEM vitamin, 0.1 mM bromodeoxyuridine, and 30 mM HEPES, pH 7.4.

Intracellular pH measurement

To examine NHE1 infection in isolated cardiomyocytes, cells were infected with pAd-NHE1. For infection, cells were simultaneously infected and placed in serum reduced medium (0.5% FBS) overnight with 10 μg/ml gentamicin. Standard infections were at a multiplicity of infection of 20, or were as indicated. Intracellular pH of cells on coverslips was measured using a PTI Deltascan spectrofluorometer. The initial rate of Na^+-induced recovery of cytosolic pH (pH_i) was measured after ammonium chloride (40 mM × 3 min) induced acute acid load using 2′,7-bis(2-carboxyethyl)-5(6) carboxyfluorescein-AM (BCECF-AM; Molecular Probes Inc., Eugene, OR, USA). Recovery was in the presence of 135 mM NaCl and was measured as described previously [27]. For some experiments cells were treated with EMD87580 of varying concentrations as indicated. EMD87580 was dissolved in PBS and the inhibitory effect of EMD87580 was documented using a two-pulse acidification assay. Cells were treated with ammonium chloride two times and allowed to recover in NaCl containing medium. One pulse was in the absence of EMD87580 and one in the presence of inhibitor. The rate

of recovery from acid load was compared ± inhibitor. Results are shown as the mean ±SE and statistical significance was determined using the Mann–Whitney *U* test.

Na^+/H^+ exchanger transfection in AP1 cells

Na^+/H^+ exchanger activity was also measured in AP-1 cells that lack an endogenous Na^+/H^+ exchanger [21]. Transfection of these cells was with LIPOFECTAMINE^{TM} 2000 Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and was used to make stable cell lines of the inhibitor resistant mutant as described earlier [22]. Transfected cells were selected using 800 μg/ml geneticin (G418) and stable cell lines for experiments were regularly re-established from frozen stocks at passage numbers between 5 and 9. Intracellular pH was measured as described above for isolated cardiomyocytes.

SDS-PAGE and immunoblotting

To confirm NHE1 expression cell lysates were made from AP-1 cells or from isolated cardiomyocytes as described earlier [22]. For Western blot analysis equal amounts of up to 100 μg of each sample were resolved on 10% SDS/polyacrylamide gels. For NHE1 detection nitrocellulose transfers were immunostained using anti-HA monoclonal antibody (Boehringer Mannheim, Laval, Que., Canada) and peroxidase-conjugated goat anti-mouse antibody (Bio/Can, Mississauga, ON, Canada). MF 20 anti-myosin antibody was from the Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA. The Amersham enhanced chemiluminescence Western blotting and detection system was used to visualize immunoreactive proteins.

Results

Figure 1 illustrates the two different types of NHE1 gene constructs used in this study. The transgene used in the construction of transgenic mice that overexpress NHE1 is shown in Fig. 1A. It consisted of full length NHE1 cDNA flanked at the 5′ end by the cardiac specific alpha myosin heavy chain promoter and with a growth hormone polyadenylation signal at the 3′ end. The 1K3R4E mutation in the calmodulin binding domain was either absent or present in the N- and K-lines respectively. Figure 1B illustrates the recombinant gene generated by insertion of NHE1 into pAdTrack-CMV followed by recombination with pADEasy1. The NHE1 cDNA had the L163F/G174S mutation and was flanked by the CMV promoter on the 5′ end and a polyadenylation signal at the 3′ end. The construct also had a GFP transcript driven by the CMV promoter to allow for easy confirmation of infection. Both constructs have HA tags at the 3′ end of the NHE1 cDNA that we have previously shown do not interfere with NHE1 expression or activity [27].

Characterization of transgenic mice overexpression of the Na^+/H^+ exchanger in the myocardium

The cardiac MHC promoter drove cardiac specific overexpression of the Na^+/H^+ exchanger. PCR analysis of genomic DNA used the specific primers (Fig. 1) to identify transgenic founder mice. Four transgenic mice were identified with the wild type Na^+/H^+ exchanger (N lines) and five with the 1K3R4E mutation (K lines). Of these, three of each was chosen after preliminary analysis. The level of expression of transgenic mice of exogenously introduced genes varied even with the same promoter. The cause of the variation is likely due to the influence of genes surrounding the location of the integrating DNA. Figure 2A examines the relative levels of expression of the 3 N-line transgenic mice. Lanes 1–3 are the low, medium and high level expressors respectively. Lane 4 is a positive control made from a cell extract of AP-1 cells transfected with the HA-tagged NHE1 cDNA. The upper panel of Fig. 2A is a short-term exposure of the film that shows that the 3 lines express increasing amounts of the protein. The lower panel is a longer exposure that more clearly shows the expression in the low N-line. These 3 lines were each chosen as either low, medium or high level expressors, based on the intensity of the immunoreactivity on Western blots. Figure 2B shows that we obtained similar results with the K-line series of mice. We found that 3-lines of mice had varying levels of Na^+/H^+ exchanger expression that could be described as low, medium or high.

Figure 2C examines the tissue specific expression of one month old N-line [28] transgenic mice. The expression of the Na^+/H^+ exchanger was specific to the heart, and there was no detectable expression in the lung, liver, kidney or spleen. A trace of expression was sometimes seen in the brain though this amounted to about 5% or less than that of the heart. Similar results were obtained with the low level expressing N-line mice (not shown). Similar, results were obtained with the K-type mice (Fig. 2D). Expression was exclusively in the myocardium.

We examined the developmental expression of the transgene in the intact myocardium. Figure 2E shows an example of a time course of expression of transgene hearts from N- and K-line mice. Expression was detected as early as 1 week post birth and continued until several months past birth. Expression continued in animals more than ½ year of age (not shown).

To confirm that the NHE1 transgene was expressed in cardiomyocytes of the myocardium, and not primarily in

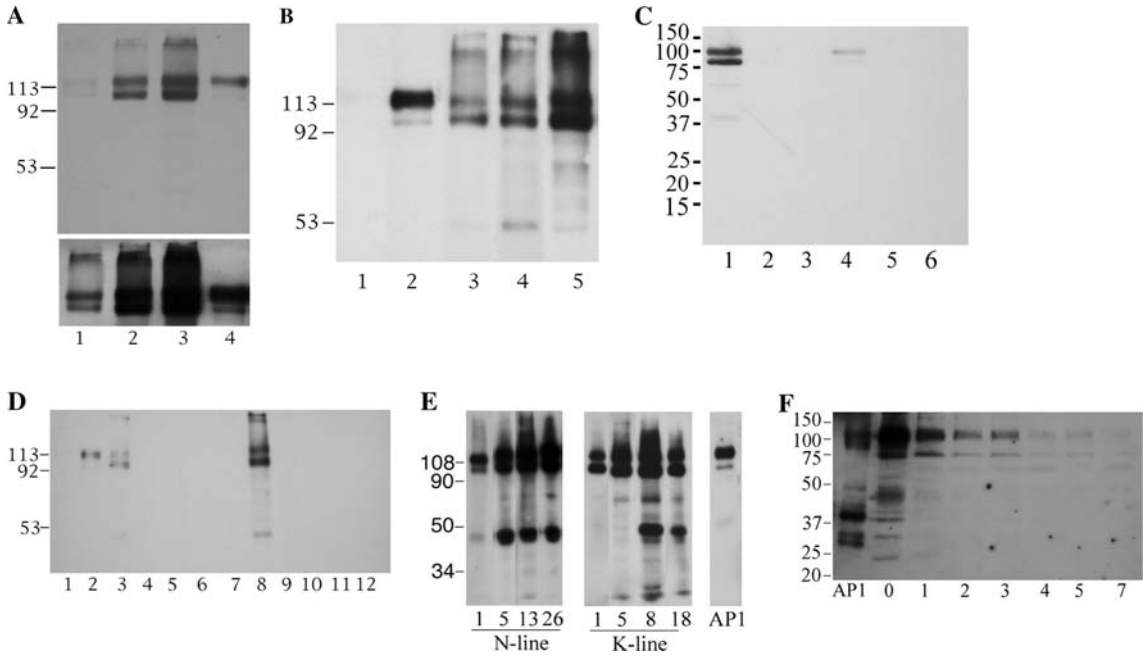

Fig. 2 Western blot analysis of transgenic mice for expression of the Na^+/H^+ exchanger. (**A**) Western blot analysis of relative levels of expression of the Na^+/H^+ exchanger in low, medium and high N-line transgenic mice. Lanes 1–3 are heart extracts immunoblotted with anti HA-antibodies. Lane 4 is a positive control from an extract of AP-1 cells transfected with the Na^+/H^+ exchanger. Upper panel is a short-term exposure while the lower panel is a long-term exposure of the Western blot. (**B**) Western blot analysis of relative levels of expression of the Na^+/H^+ exchanger in low, medium and high K-line transgenic mice. Lane 1, heart extract of control mice. Lane 2, positive control made from AP-1 cells transfected with HA-tagged Na^+/H^+ exchanger. Lanes 3–5, heart extracts of three independent N-line transgenic mice. (**C**) Western blot of tissue specific expression of the N-line [28] transgenic mice. Lanes 1–6 are extracts of heart, lung, kidney, brain, spleen and liver respectively from 1-month old mice. The expression of the Na^+/H^+ exchanger was specific to the heart, and there was no detectable expression in the lung, liver, kidney or brain. Similar results were obtained with the low level expressing N-line mice (not shown). (**D**) Tissue specific expression of K-line mice. Lanes 3–7 are from a low expressing K-line. Lane 1 is an extract of a non-transgenic heart. Lane 2 is a positive control, a cell extract from AP-1 cells transfected with HA-tagged Na^+/H^+ exchanger. Lanes 3–7 are extracts from the heart, lung, liver, kidney and brain respectively. Lanes 8 – 12 are extracts of heart, lung, liver kidney and brain respectively from a high level expressing K-line. (**E**) Time course of expression of NHE1 transgene in the intact myocardium. Hearts extracts were made from intact myocardium of either N-line [28] or K-line [28] mice at the age indicated (in weeks). AP1 is a positive control made from AP1 cells transfected with HA-tagged NHE1. (**F**) Time course of expression of the NHE1 protein in isolated cardiomyocytes made from N-line transgenic mice 1 month of age. Isolated cardiomyocytes were made and cultured for the time indicated as described in the ''Materials and Methods''. Equal numbers of cells were plated and Petri dishes were harvested at various times after plating cells. Cell lysate proteins were analyzed for NHE1 presence by Western blotting against the HA tag

other cell types, we cultured cardiomyocytes from 1-month old N-line [28] transgenic mice. Culturing cardiomyocytes from adult mice was not successful (data not shown). Cells from 1-month old mouse hearts were then kept in culture for up to 7 days. Western blotting was used to examine the presence of the HA-tagged NHE1 protein. The results are shown in Fig. 2F. At day 0, immediately after the cardiomyocytes were made, the NHE1 protein was apparent. Surprisingly, the abundance of the protein declined greatly with time. It was relatively abundant 1 day after cardiomyocyte preparation, but declined rapidly such that after 3 days it was barely detectable. Maintaining cells in 15% fetal bovine serum (shown) as opposed to 10% bovine growth serum made little difference in the time course of the disappearance of NHE1 protein (not shown). Figure 3A–D illustrates the morphology of the cardiomyocytes maintained in culture. When initially prepared, the cells were smaller and did not adhere well to the Petri dishes (Fig. 3A). However by the third day after preparation (Fig. 3B) the cells spread, were more ''myocyte-like'' in morphology and beat occasionally. The cells spread more and maintained this morphology and continued to contract even one week after preparation (Fig. 3D) or for longer times (not shown).

Characterization of NHE1 Phenotype in Transgenic Mice

We examined the survival rate of transgenic mice with the NHE1 transgenes. The results are shown in Fig. 4. There was no significant mortality of control mice over the 8 month period (not shown). Figure 4A shows that in N-line mice there was a significant mortality associated with expression of NHE1 in the myocardium, especially

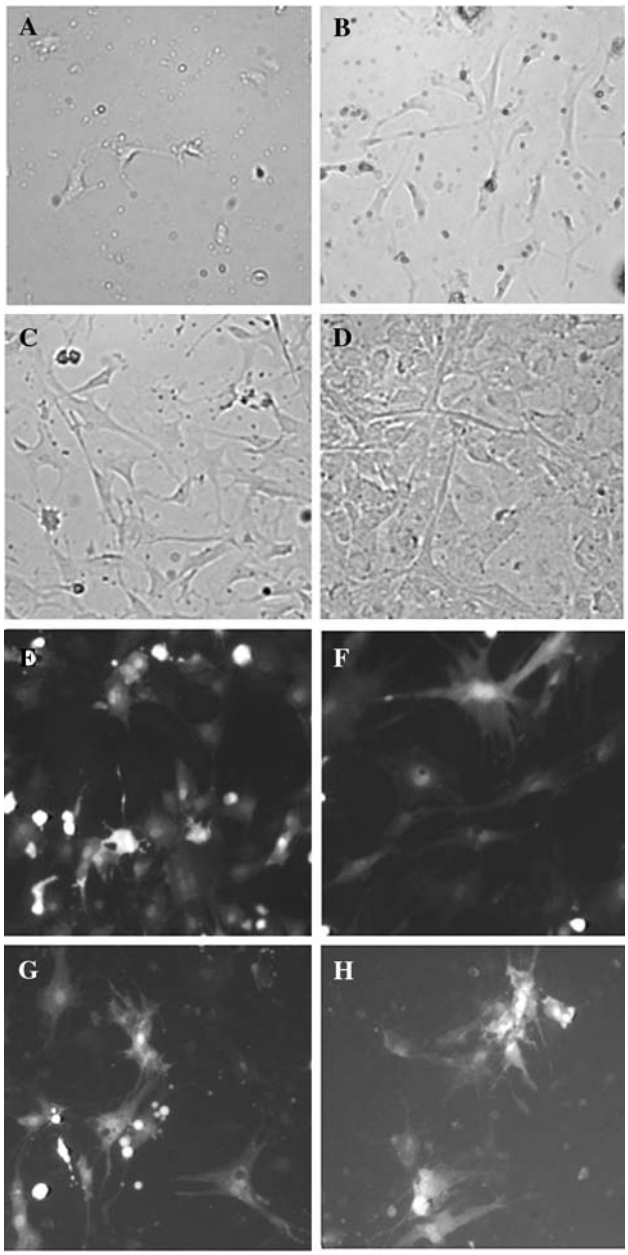

Fig. 3 Isolated cardiomyocytes from mouse transgenic hearts. Isolated cardiomyocytes were made and cultured as described in ''Materials and Methods'' and were maintained in culture for the indicated times. (**A–D**) Light microscopy of isolated cardiomyocytes maintained in culture for 2, 3, 4 and 7 days (respectively). (**E–H**) GFP reporter fluorescence of isolated cardiomyocytes infected with pAd-NHE1. Cells were examined 1, 2, 3 and 4 days after viral transfection respectively

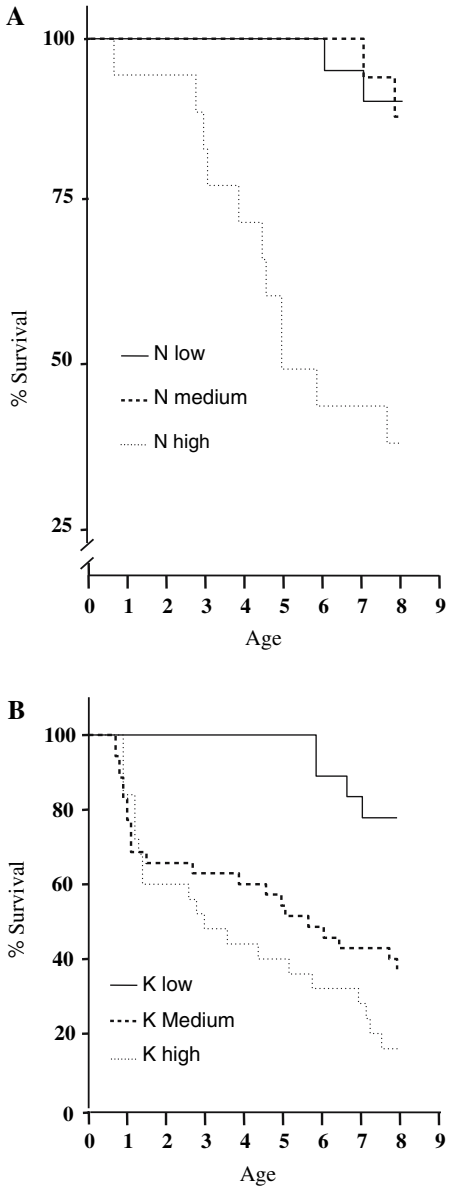

Fig. 4 Kaplan Meier analysis of different lines of transgenic mice expressing either the wild-type Na^+/H^+ exchanger (N-line) (**A**) or the mutant (K-line) Na^+/H^+ exchanger transgene (**B**). The analysis is shown for the three types of each line for up to 8 months of age. There was no significant mortality of control mice during this time

when expression was at high levels. Similar results were found with the K-line mice except that this form of the Na^+/H^+ exchanger protein caused a more severe effect on survival (Fig. 4B), especially in the line expressing higher levels of the protein.

The effects on the mice appeared to be intermittent, with some mice being severely affected and others unaffected (not shown). The reason for this is not yet clear at this time and will be the subject of future investigations. We examined K-line transgenic mouse heart in intact mice to determine the nature of the defects (Fig. 5). M-mode echo analysis revealed ventricle dilation in the transgenic mice in both the systolic and diastolic phase. Comparison of a control heart with that of a severely compromised transgenic showed that left ventricular diastolic volume had increased dramatically with the appearance of a hypertrophy or a dilated cardiomyopathy. Further confirmation of this phenotype is shown in Fig. 6. Transgenic mice showed

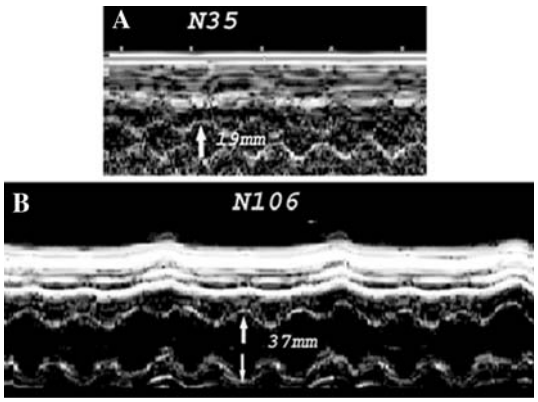

Fig. 5 Echocardiographic analysis of mouse hearts. M-mode echocardiograms using a Philips 5500 cardiac ultrasound system with a 12 MHz phased array transducer. The system permits two-dimensional echocardiographic studies using both pulsed and color Doppler intra- and extracardiac blood flow interrogation. (**A**) Example of control heart showing a normal left ventricular diastolic volume. (**B**) Example of age matched K-line heart

an enlargement of the heart in general overall size and in cross sectional area.

Characterization of mutant L163F/G174S Na^+/H^+ exchanger

We mutated the NHE1 isoform of the Na^+/H^+ exchanger so that it was resistant to inhibition. To confirm that the mutation was successful and that the mutant Na^+/H^+ exchanger retained activity we stably transfected AP-1 cells with the mutated cDNA and carried out two pulse activity assays as described in ''Materials and Methods''. The mutant was markedly more resistant to inhibition by the NHE1 inhibitor HOE 694. The IC50 changed from 0.15 μM to 37.1 μM, a change of over 200-fold in resistance (not shown).

Characterization of NHE1 Expression via Adenoviral Transfection of Cardiomyocytes. To determine if the adenoviral system could be used to transfect neonatal isolated cardiomyocytes we constructed an expression vector with the inhibitor resistant NHE1 as described in the ''Materials and Methods''. Western blot analysis (Fig. 7A) demonstrates NHE1 expression in primary cultures of neonatal cardiomyocytes when infected with adenovirus. The lane AP-1 refers to a positive control made by transfection of AP1 cells with a HA-tagged NHE1. Lanes 1 and 2 are cell extracts from isolated cardiomyocytes transfected with the pAd-NHE1 adenovirus. Both the glycosylated and the deglycosylated form of the NHE1 protein are visible. Lane 3 shows a cell extract from cells infected with a control adenovirus, not containing the HA-tagged NHE1 protein. Figure 7B is a Western blot of the same samples probed with anti-myosin antibody demonstrating that approximately equal amounts of sample were present. Figure 7C is a Western blot examining the time course of expression of the NHE1 protein after the initial infection at

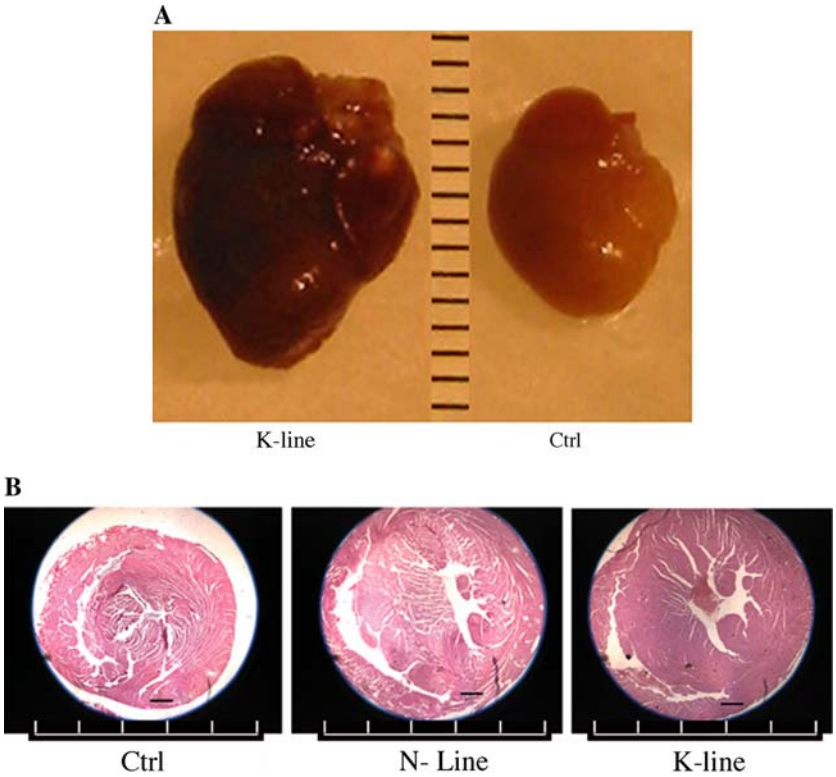

Fig. 6 Comparison of hearts of transgenic and control mice. (**A**) Hearts from control and K-line transgenic mice. Grid indicates 1 mm increments. (**B**) Transverse sections of control (Ctrl) and N-line and K-line transgenic mice at 2.5 months of age. Black inset bar is 0.5 mm and white grid indicates 1 mm increments

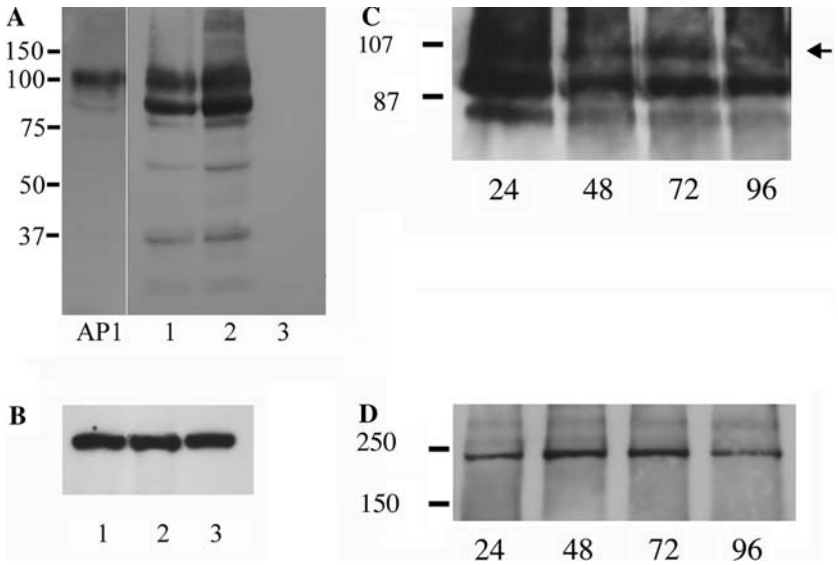

Fig. 7 Characterization of expression of the Na^+/H^+ exchanger in isolated cardiomyocytes transfected with adenovirus containing NHE1 protein (pAdNHE1). Isolated cardiomyocytes were made from neonatal rat hearts as described in the ''Materials and Methods''. Equal numbers of cells were then infected with pAdNHE1 containing the HA-tagged inhibitor resistant NHE1 protein. Western blots of cell extracts were blotted with anti-HA antibody. (**A**) ''AP1'' is a positive control made from AP1 cells transfected with a HA-tagged NHE1 protein. Lanes 1 and 2 were extracts from isolated cardiomyocytes infected with pAdNHE1, which contains the HA-tagged inhibitor resistant NHE1 isoform of the Na^+/H^+ exchanger. Lane 3 was from cells infected with a control adenovirus which had no NHE1. (**B**) Western blot of lanes 1–3 of 7A re-probed with antibody to myosin (MF-20). (**C**) Time course of expression of NHE1 protein in isolated cardiomyocytes infected with pAdNHE1. Sixty mm dishes of cells were infected with the adenovirus containing NHE1 and the time course of expression was examined by Western blotting against the anti-HA tag. Cells were infected with a multiplicity of infection of 20 and were examined at 24–96 h post infection. Arrow indicates the mobility of the fully glycosylated NHE1 isoform. (**D**) Samples of Fig. 7C probed with antibody to myosin (MF-20)

a multiplicity of infection of 20. NHE1 expression was present as early as 24 h after infection and continued 96 h post infection. For this experiment only one 60 mm dish of cardiomyocytes was used for harvest and assay at each time point. Protein concentrations declined slightly with time of infection, likely accounting for the slight decline in signal strength with time. Both the glycosylated and the deglycosylated form of the NHE1 protein are visible. Figure 7D is a Western blot using anti- myosin antibodies and demonstrates that comparable amount of protein is present in the samples.

To confirm that the pAd-NHE1 had infected the cardiomyocytes we examined fluorescence of the cells. In addition to the NHE1 cDNA, the adenovirus pAd-NHE1 contains a GFP marker that allows confirmation of the infection. Figure 3E–H illustrates the fluorescence of the cells 1–4 days after viral infection. In all cases the cells contained the GFP protein and over 95% of the cells were infected.

To confirm that we had expressed the inhibitor resistant mutant in isolated cardiomyocytes we measured the activity of the Na^+/H^+ exchanger in either control isolated cardiomyocytes or cardiomyocytes that had been transfected with pAD-NHE1 adenovirus for 24 h at a MOI of 20. The activity of the Na^+/H^+ exchanger was measured in varying concentrations of the Na^+/H^+ exchanger EMD 87580 from 0.3 μM to 300 μM. The results are shown in Fig. 8. In uninfected cells, EMD 87580 inhibited the endogenous Na^+/H^+ exchanger with an IC_{50} of 0.3 μM. In cells infected with inhibitor resistant NHE1, the IC_{50} was 30 μM, demonstrating that the inhibitor resistant protein was expressed in these cells. For future experiments a concentration of 10 μM was used which inhibited the endogenous protein completely, and only would have a minor effect on exogenous NHE1.

To characterize the inhibitor resistant NHE1 protein in primary cultures of cardiomyocytes we assayed the activity of the Na^+/H^+ exchanger in the presence or absence of the NHE1 inhibitor EMD87580. The results are shown in Fig. 9. In either mock infected mouse cardiomyocytes, or cardiomyocytes infected with adenovirus without the NHE1 gene, the activity of the Na^+/H^+ exchanger was reduced to negligible levels. In cardiomyocytes infected with adenovirus containing the inhibitor resistant NHE1, Na^+/H^+ exchanger activity was retained after treatment with 10 μM EMD87580. Since the Na^+/H^+ exchanger activity initially measured was a combination of the endogenous NHE1 protein and the protein introduced by the adenovirus,

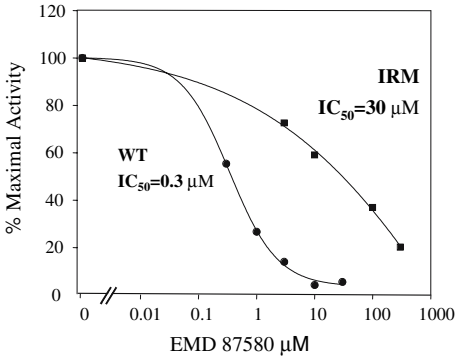

Fig. 8 Characterization of Na^+/H^+ exchanger activity in isolated cardiomyocytes infected with pAdNHE1. Cells were uninfected or infected with pADNHE1 at an MOI of 20 for 24 h. The cells were then subjected to two pulse Na^+/H^+ exchanger activity assays and the activity of the exchanger in the second pulse in the presence of the inhibitor, was compared to that of the first pulse in the absence of the inhibitor EMD 87580. Circles, uninfected cardiomyocytes. Squares, cells infected with pAdNHE1. Wt, wild type Na^+/H^+ exchanger activity in uninfected cardiomyocytes; IRM, inhibitor resistant mutant activity of infected cardiomyocytes

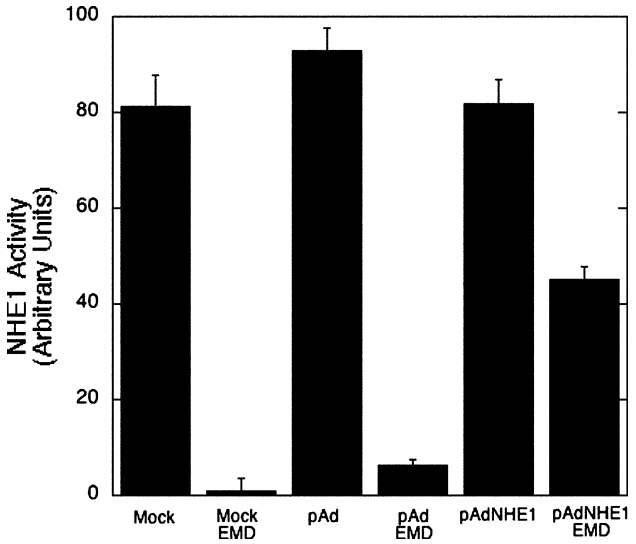

Fig. 9 Na^+/H^+ exchanger activity of isolated cardiomyocytes infected with adenovirus containing NHE1 protein. Isolated cardiomyocytes were made and infected with NHE1 containing adenovirus as described in Fig. 7. Cells were then assayed for NHE1 activity using a two pulse assay, with the second pulse ±10 μM EMD87580. The activity of the second pulse relative to the first is displayed for each bar. pAd, cardiomyocytes infected with adenovirus without an NHE1 insert; pAdNHE1, infected with adenovirus containing NHE1 insert; EMD, assayed in the presence of 10 μM EMD87580; Mock, mock infected cardiomyocytes

the decline in activity due to 10 μM EMD87580, is likely due to decreased activity of the endogenous Na^+/H^+ exchanger. The results showed that in cells infected with pADNHE1, over half the Na^+/H^+ exchanger activity was due to introduction of the exogenous gene.

Discussion

The NHE1 isoform of the Na^+/H^+ exchanger is responsible for removal of excess intracellular protons in the myocardium. It removes one intracellular proton in exchange for one extracellular sodium. It has been implicated in the damage that occurs to the myocardium during ischemia and reperfusion and plays an important role in heart hypertrophy [2, 3]. The level of expression and activity of the Na^+/H^+ exchanger has been shown to vary in the myocardium in various disease states such as hypertension, hypertrophy, or in the diabetic myocardium [29]. For example, the activity of NHE1 is elevated in human patients with end stage heart failure [30], and NHE1 levels increase in isolated cardiomyocytes subjected to chronic acidosis [19]. Hearts subjected to ischemia [18] also show increases in NHE1 levels and ischemia and reperfusion also activates the protein kinases that regulate NHE1 activity [20].

It was therefore of interest to try to develop animal models that mimic the increase in NHE1 levels and activity that occur in the pathophysiological state. In this report we examine two models for increasing NHE1 levels and activity in the mammalian myocardium and show the initial results of the effects of this increased expression and activity on intact animals. We initially developed two transgenic models of expression of the Na^+/H^+ exchanger. In one, we overexpressed the control protein (N-line), while in a second we overexpressed a mutant Na^+/H^+ exchanger protein that has slightly elevated activity (K-line) due to a mutation of the calmodulin binding domain [23]. Several lines of each type were made that expressed different levels of the NHE1 protein. The varying level of expression was likely due to the influence of areas of the genome that surrounded where the transgene was integrated. For each type of transgene, we arbitrarily referred to the mouse lines as low, medium and high. The change in activity of the NHE1 protein in isolated cardiomyocytes from these transgenic hearts was an increase of approximately two and three-fold for the N-line [28] and K-line (medium) mice respectively (not shown).

Use of the alpha myosin heavy chain promoter resulted in specific expression of the NHE1 protein in the intact myocardium. This occurred for various lines of mice with the two different transgenes. NHE1 was specifically present in the myocardium and not in any other intact tissue (Fig. 2C,D). The expression occurred at an early age and continued throughout the measured life of the mice. Our conclusion of these observations is that, in the intact animal, NHE1 overexpression in the myocardium was specific and provides a useful model for studying the role of this protein in myocardial disease.

We also confirmed that the NHE1 transgene was expressed specifically in primary cultures of one-month-old

cardiomyocytes, as opposed to other cell types in the myocardium. However, in the process of this determination we also demonstrated that expression from the alpha myosin heavy chain promoter declined with time. By the fourth day after preparation of isolated cardiomyocytes, there was little if any expression of the exogenous Na^+/H^+ exchanger protein. The same phenomenon occurred with either the wild type NHE1 gene or the mutant gene. Changing the standard culture medium from 10 to 15% serum did not prevent the decline in expression (not shown). The reason why expression declined is likely that the appropriate stimuli for the alpha myosin heavy chain promoter were not present in the culture media of the isolated cardiomyocytes. Thyroid hormone levels might be critical in this regard [31] and future experiments will investigate the effects of thyroid hormone on this promoter. These results suggest that while expression in the intact animal works well, studying cardiomyocytes from transgenic animals with this promoter should be limited to either freshly made cardiomyocytes, or to relatively short periods of time after initial cell preparation. Future experiments will attempt to optimize culture conditions to determine if expression from the alpha myosin heavy chain promoter can be retained.

We characterized the effects of expression of the transgenes on survival in the mice. There appeared to be a time and dose dependent effect of the transgenes. In the N-line mice, expression of low or medium levels of the transgene had little effect. However the high levels of expression of the transgene caused a decrease in the survival of the mice. In the K-line mice the effect of expression of the transgene was more severe. With the high line, mortality was approximately 80% after 8 month. Even the low expressing line showed 20% mortality after 8 months. While we have not yet investigated the exact cause of death in detail, it was apparent that many of the mice were dying from a cardiac specific defect. For reasons that are not yet clear at this time, some mice remained relatively healthy while others of the same line and expression level, possessed severe defects. Figure 5 illustrates an echocardiogram from hearts of a control mouse and from a K-line mouse with an apparent and severe defect. Left ventricular diastolic volume increased dramatically with the appearance of a hypertrophy or a dilated cardiomyopathy. The exact series of events that lead from the expression of the transgene to the defect, and the cause of the irregular phenotype will be the subject of future investigations. Examples of the effects on the morphology of the heart are shown in Fig. 6. Hearts from transgenic mice were larger in both overall size, diameter and intraventricular septal wall thickness.

Since many investigations in cardiac biology have studied isolated cardiomyocytes, we developed a system for overexpression of the Na^+/H^+ exchanger in isolated heart cells. Cardiac myocytes possess their own NHE1 isoform of the Na^+/H^+ exchanger, so to distinguish activity and effects of an exogenous protein, we made a mutant Na^+/H^+ exchanger that was resistant to NHE1 inhibitors. The Leu163Phe/Gly174Ser mutation caused an increase in the IC50 for inhibition of over 200-fold. An adenovirus containing NHE1 demonstrated that infection of isolated cardiomyocytes results in stable expression of the NHE1 gene. The infection of the cells was almost complete by the adenovirus and myocytes maintained their general morphology and contractility in culture after infection. It was possible to eliminate endogenous NHE1 activity by inclusion of NHE1 inhibitors. The adenoviral expression system will thus allow the study of the effects of NHE1 overexpression in primary cultures of isolated cardiomyocytes. Future experiments may examine aspects of NHE1-related pathologies such as calcium fluxes in cells overexpressing the NHE1 protein.

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