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Elevated expression of activated Na^+/H^+ exchanger protein induces hypertrophy in isolated rat neonatal ventricular cardiomyocytes

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Abstract The plasma membrane protein the Na^+/H^+ exchanger isoform1 (NHE1) has been implicated in various cardiac pathologies including ischemia/reperfusion damage to the myocardium and cardiac hypertrophy. Levels of NHE1 protein and activity are elevated in cardiac disease; however, the mechanism by which these factors contribute to the accompanying hypertrophy in the myocardium is still not clear. To investigate the mechanism of NHE1-induced hypertrophy in the myocardium we constructed two adenoviral vectors expressing either wild type NHE1 protein or a constitutively active NHE1 protein. Infection of neonatal rat ventricular cardiomyocytes (NRVM) resulted in elevated expression of both wild type NHE1 or constitutively active NHE1. Only expression of activated NHE1 protein resulted in an increase in cell size and in an increase in protein synthesis in isolated cardiomyocyte cells. The results demonstrate that expression of activated NHE1 promotes cardiac hypertrophy in isolated cardiac cells and that simple elevation of levels of wild type NHE1 protein does not have a significant hypertrophic effect in NRVM. The results suggest that regulation of NHE1 activity is a critical direct effector of the hypertrophic effect induced in the myocardium by the NHE1 protein.

Keywords Adenovirus · Cardiomyocyte · Cardiac hypertrophy · Intracellular pH · Na^+/H^+ exchanger

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Abbreviations

HA	Hemagglutinin
NHE	Na^+/H^+ exchanger
NRMVs	Neonatal rat ventricular cardiomyocytes

The Na^+/H^+ exchanger (NHE) is a ubiquitously expressed plasma membrane glycoprotein that regulates intracellular pH by extruding one intracellular H^+ in exchange for one extracellular Na^+ [1, 2]. There are several isoforms of NHE1 (NHE, isoform 1), however, NHE1 is the only plasma membrane isoform of the ten isoforms described that is present in cardiac cells [3–5]. NHE1 is involved in myocardial disease. Increased NHE1 activity plays a key role in mediating the damage that occurs during ischemia/reperfusion injury (I/R injury) [2, 6, 7] and NHE1 activity is an important mediator of cardiac hypertrophy [8]. Inhibitors of NHE1 [9] have been tested for use in treatment of cardiac disease. As of now, their use has been very successful in animal models, but not so in human trials, partly because of detrimental side effects (reviewed in [10, 11]).

Cardiac hypertrophy is the natural response of the myocardium to various stressors, including neurohormonal stimuli, hemodynamic overload, and injury [12]. Continued stress progresses to pathological hypertrophy, a major risk factor for cardiac related deaths and commonly precedes the loss of cardiomyocytes, the development of fibrosis, and heart failure [13]. Numerous signaling pathways have been implicated in stress-induced remodeling of the heart, including intracellular calcium homeostasis, several calcium-dependent signaling molecules, and mitogen-activated protein kinases [14, 15]. The signaling pathways leading to this are very complex, but recent studies have

shown that NHE1 activity is increased in several in vivo and in vitro models of cardiac hypertrophy [3, 8, 16]. Regulation of NHE1 expression varies depending on environmental stimuli [17] and increased NHE1 expression and activity occurs in different cardiac pathologies [2, 16, 18–22]. It is of relevance that recent studies have shown that NHE1 activity is increased in in vivo and in vitro models of cardiac hypertrophy [16, 20, 23]. NHE1 inhibition has also been demonstrated to be beneficial in the prevention/regression of several models of myocardial hypertrophy [16, 20, 23–27].

The NHE1 protein is normally only active when intracellular pH decreases. When cells are at neutral intracellular pH (pH_i), NHE1 is essentially inactive. When pH_i drops, NHE1 activity increases [2, 28]. Hormonal stimulation of NHE1 activity changes the set point of NHE1 so that the protein is more active at more alkaline pH's [4, 28]. Because the unstimulated protein is essentially inactive at resting physiological pH's [28], we have questioned whether elevations of the levels of non-activated NHE1 protein are detrimental to the myocardium. To test whether activated or non-activated protein affect the pathogenesis of myocardial hypertrophy in intact animals we recently made transgenic models of mice overexpressing NHE1 in the myocardium. We showed [29] that in intact mice, expression of activated NHE1 caused significant increases in heart weight to body weight, and other indices of cardiac hypertrophy. However, in this type of model NHE1 was expressed throughout development of the myocardium and during early heart growth. There may have been secondary effects that caused cardiac hypertrophy and thus it remains unclear as to whether the results are due to long-term secondary effects of NHE1 expression, or whether cardiomyocyte hypertrophy is more directly caused by increased expression of an activated NHE1 protein in cardiomyocyte cells. 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 examined direct effects of the activated protein in isolated NRVMs. Two adenoviruses overexpressing either a wild type or activated NHE1 protein were used in our study. Our results demonstrate that expression of wild type NHE1 protein alone is not hypertrophic; however, expression of an activated NHE1 protein induces hypertrophy in isolated NRVMs.

Materials and methods

Materials

BCECF-AM was purchased from Molecular Probes (Eugene, OR). Collagenase Type 2 was from Worthington Biochemical Corporation (Lakewood, NJ) and other chemicals were of analytical grade and were purchased from Fisher Scientific (Ottawa, ON) or Sigma (St. Louis, MO). EMD87580 was a gift of Dr. N. Beier of Merck KGaA, Frankfurter, Germany. Anti-HA (12CA5) was from Roche Applied Science (Laval, Quebec). Anti-Na^+/H^+ exchanger isoform1 was from BD Biosciences Pharmingen (San Diego, CA).

Tissue culture procedures

Mutant plasmids were transfected in the AP1 cell line, which does not express endogenous active NHE1. AP-1 is a mutant cell line derived from Chinese hamster ovarian cells (CHO) by the proton suicide technique [30]. Stably transfected cells were established using LIPOFECTAMINE™ 2000 Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) as described earlier [31]. NHE1 with an hemagglutinin (HA) tag was expressed using the pYN4+ plasmid as described earlier [32, 33]. pYN4+ plasmid encoded a neomycin resistance gene, which allowed the selection of transfected cells using geneticin (G418) antibiotics. NHE1 with an HA tag was expressed using the pYN4+ plasmid as described earlier [32, 33].

Preparation and characterization of isolated cardiomyocytes

Primary cultures of cardiomyocytes were prepared from 5- to 6-day-old neonatal Sprague–Dawley rat heart ventricles as described earlier [22]. After 48 h they were infected with adenoviruses at a multiplicity of infection of 20 where indicated. Cell area was measured from digitized images of NRVMs [34]. The cells were plated at an average density of 1×10^4/35-mm, which allowed for clear distinction of cells. After 24 h of infection/treatment, cells were washed 2× with PBS. Digitized images were gathered using a Photometrics $CoolSNAP_{fx}$ camera equipped to a Leica DMIRB microscope and cell area was measured using Image ProPlus Version 4.5. At least 50 cells were averaged from three dishes and represented as one n value [34].

Construction and use of adenoviruses

Adenoviral vectors containing HA tagged-wild type NHE1 resistant to inhibition (IRM) or HA tagged-activated NHE1 resistant to inhibition (K-IRM) were made as previously described [35]. The IRM mutation was created by mutating $Leu^{163}Phe/Gly^{174}Ser$ in NHE1 as described earlier [36]. This double mutation, $Leu^{163}Phe/Gly^{174}Ser$ in NHE1, has been shown to increase the resistance to inhibition and conferred a 100-fold increase in resistance to inhibition [36]. For the K-IRM gene, the $pYN4^+$ plasmid was

mutated on the NHE1 protein cytoplasmic regulatory domain at the following sites Lys^{641}, Arg^{643}, Arg^{645}, and Arg^{647} (mutated to Glu residues) resulting in a activated NHE1. The mutation causes an alkaline shift in NHE1 pH dependency [37, 38], which we have earlier demonstrated [29, 38, 39]. To make the activated NHE1 cDNAs resistant to inhibition, an *Age*I-*Spe*I fragment was replaced from the IRM. Therefore, the K-IRM contained the $Leu^{163}Phe$/$Gly^{174}Ser$ mutation of the membrane domain and the mutations on the NHE1 protein cytoplasmic regulatory domain at the following sites Lys^{641}, Arg^{643}, Arg^{645}, and Arg^{647} (mutated to Glu residues). IRM and K-IRM were cloned into a modified adenovirus as previously described [35]. The transfection efficiency was >99% and was monitored by green fluorescent protein (GFP) expression, which was additionally expressed by all adenoviral vectors. In a separate series of experiments (not shown) we measured NHE activity of adenovirus infected cells, while treating with NHE inhibitor and confirmed we were expressing exogenous NHE1 that was resistant to EMD87580, an NHE1 inhibitor [36].

Protein synthesis rate measurements of neonatal rat ventricular cardiomyocytes

Protein synthesis rate measurements were measured via [^{3}H] phenylalanine incorporation. Cells were plated in 12-well plates at a density of 3.3×10^5. [^{3}H] Phenylalanine (Amersham Biosciences, Piscataway, NJ) (1 μCi/ml) was added four hours after viral infection. Cells were left for 24 h at 37°C in 5% CO_2. Cell suspensions were prepared as previously described [40]. In brief, cells were washed 3× with ice-cold 1× PBS and than incubated at 4°C for 1 h in 10% trichloroacetic acid. The precipitates were washed twice with 95% ethanol, dissolved in 1 M NaOH, scraped and neutralized with 1 M HCl. Radioactivity was counted in a liquid scintillation counter. Protein synthesis was corrected with DNA content measured by hoescht 33258 as previously described [41]. Hoescht 33258 was diluted to 0.1 μg/ml in ddH_2O containing Tris pH 7.4, EDTA, NaCl and added to cell suspension. The amount of DNA present was measured through fluorescence, using a PTI Deltascan spectrofluorometer (Photon technology international, London, ON). The excitation filter was set at 360 nm and the emission filter was set at 450 nm. Final results were expressed as counts per minute/μg of DNA [41].

Western blot analysis

For immunoblot analysis of NHE1 protein in NRVMs or AP-1 cells, cell lysates were made essentially as described earlier [22, 42] and contained a protease inhibitor cocktail to prevent degradation of the NHE1 protein [43]. Total protein was quantified using the Bio-Rad DC 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-NHE1 or anti-HA antibody over night and β-tubulin antibody, a loading control. To visualize and quantify immunoreactive proteins we used a Li-COR fluorescence labeling and detection systems (LI-COR Biosciences, Lincoln, NE).

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

Increased sympathetic activity, mediated by agonists such as PE, has been implicated in the development of cardiac hypertrophy in animal models [16, 44] and in humans [45]. To test if increased activity of Na^+/H^+ exchanger was a contributing factor in the development of cardiac hypertrophy in isolated cardiomyocytes, we developed expression vectors that contained either a wild type, or an activated form of the NHE1 protein (Fig. 1). We have previously demonstrated that the mutation Lys^{641}, Arg^{643}, Arg^{645}, and Arg^{647} to Glu residues causes an alkaline shift in NHE1 pH dependency activating the NHE1 protein [29, 38, 39]. The protein we engineered for expression in isolated cardiomyocytes contained a HA tag. The pAdTrack plasmids (IRM-pAdTrackCMV and K-IRMpAdTrackCMV) contained the NHE1 cDNA and were used to create the adenoviruses for infection of cardiomyocytes. To confirm that we had correctly constructed the exogenous tagged NHE1

Fig. 1 Adenovirus constructs used for the expression of NHE1 in neonatal rat ventricular cardiomyocytes. The IRM adenovirus contains HA tagged-wild type NHE1 resistant to NHE1 inhibitors. The IRM mutation is L163F/G174S. The K-IRM adenovirus contains HA tagged-active NHE1 resistant to NHE1 inhibitors plus a further mutation that activates the NHE1 protein. The notation 1K3R4E indicates that the DNA contained the activating mutation of lysine (K) 641, arginine (R) 643, 645, and 647 to glutamic acid (E) residues and is indicated by (+). *CMV* cytomegalovirus promoter, *GFP* green fluorescent protein, *HA* hemagglutinin tag, polyadenylation (*PA*) signal, *LTR* long terminal repeat

protein we used western blotting with anti-HA antibodies. Figure 2a illustrates the results. A control lane (pYN4+) contained a cell extract from AP-1 cells that were transfected with plasmid containing HA-tagged NHE1 protein. As expected, it contained immunoreactive NHE1 protein. Typically a fully glycosylated NHE1 protein (110 kDa) is seen plus a 90 kDa partially or de-glycosylated NHE1 protein [32]. Transfection of AP-1 cells with the pAd-TrackCMV protein (without the NHE1 insert), as expected, did not produce any protein immunoreactive with anti-HA antibody. Conversely, transfection of AP-1 cells with either the wild type NHE1 protein (IRM) or the activated NHE1 protein (K-IRM) produced HA-tagged NHE1 protein that was detected in our immunoblot analysis. The results demonstrated that the NHE1 containing plasmids produce the expected NHE1 protein specifically from the HA-tagged NHE1 insert.

Prior to the characterization of our IRM and K-IRM adenoviruses, we identified the ideal MOI and duration of viral infection in NRVMs. This was done to insure near 100% efficiency of the adenoviral infections, maintenance cell viability and optimal time to harvest infected cells. Figure 2b illustrates representative western blots of NRVMs infected for various times with the K-IRM adenovirus at an MOI of 10, 20, and 30. NHE1 expression was detected using anti-HA antibody and was present 24 h after infection. Expression continued 48 h post infection at a reduced rate. Both the glycosylated and the deglycosylated form of the NHE1 protein were visible at a MOI of 20 following 24 h of infection. Similar experiments were conducted using the IRM adenovirus and a MOI of 20 for 24 h also gave the best conditions (data not shown). NRVMs were infected with the same adenovirus but not containing NHE1 at an MOI of 20 for 24 h to confirm the specificity of the HA-tagged antibody. No immunoreactivity was detected. In all subsequent experiments, NRVMs were infected with adenoviruses using an MOI of 20 for 24 h.

In order to determine the level of total NHE1 protein in the NRVM we immunoblotted with antibody against the NHE1 protein itself (as opposed to the tag). The results are shown in Fig. 2c—the upper panel is an example of a blot and the lower panel illustrates a summary of the results. There was a large, approximately tenfold increase in the level of total NHE1 protein which was equivalent with infection with the IRM and K-IRM expressing adenoviruses. The size of the fully glycosylated NHE1 protein was slightly greater in the IRM and K-IRM infected cells, this was likely due to the presence of the triple HA-tag. This analysis estimated the levels of both the fully glycosylated and partial or de-glycosylated NHE1 protein. Previous studies have suggested that the fully glycosylated form of NHE1 is the main form present on the cell surface [46]. An analysis of the relative levels of fully glycosylated NHE1 protein showed that the IRM and K-IRM infected cells had approximately a fourfold elevation in protein levels (not shown).

We also confirmed that the exogenous HA-tagged protein was expressed in isolated cardiomyocytes (Fig. 2d). As expected, infection of cardiomyocytes with adenovirus that did not contain HA-tagged NHE1 protein, did not yield any immunoreactive NHE1 protein. Infection with either IRM or K-IRM adenovirus resulted in the immunoreactive protein being present in the cell lysates of these NRVMs. The results confirmed that NHE1 was successfully expressed in isolated cardiomyocytes. Both the control and experimental adenoviruses expressed a GFP protein. Light microscopy demonstrated that the rate of infection was >99% (not shown).

To evaluate the effects of varying types of NHE1 protein expression on the degree of cardiac hypertrophy in vitro, hypertrophic indices were measured in NRVMs infected with GFP, IRM, or K-IRM adenoviruses. Cells were infected with IRM, K-IRM, or GFP-control adenoviruses and cell area and protein synthesis were monitored. Figure 3 illustrates that only NRVMs infected with the K-IRM adenovirus showed a significant increase in cell area versus NRVMs infected with the control adenovirus or IRM adenovirus.

We also examined proteins synthesis of NRVMs infected with adenovirus expressing NHE1 protein. Protein synthesis is another index of cardiac hypertrophy. Figure 4 shows that only NRVMs infected with the K-IRM adenovirus in had a significant increase in protein synthesis ($P < 0.005$ for K-IRM vs. GFP, IRM). When NRVMs were infected with the IRM adenovirus they did not show a significant increase in protein synthesis versus NRVMs infected with the GFP adenovirus (Fig. 4).

Discussion

Studies have shown that increased levels of NHE1 protein, message, and activity are reported in several in vivo and in vitro models of cardiac diseases [16, 18–21, 24]. Specifically, the contribution of NHE1 to cardiac hypertrophy is becoming increasingly of clinical interest and preclinical studies have had promising results in preventing cardiac hypertrophy via use of NHE1 inhibitors. NHE1 is intrinsically regulated such that it has a set point and is inactive at physiological pH's near 7.4 [28]. This would mean that simple elevation of the level of the protein alone might not be very deleterious to the myocardium, as the protein may be inactive except when recovering from acid load or in pathological situations that change the set point. Complicating this viewpoint is the fact that NHE1 also has other

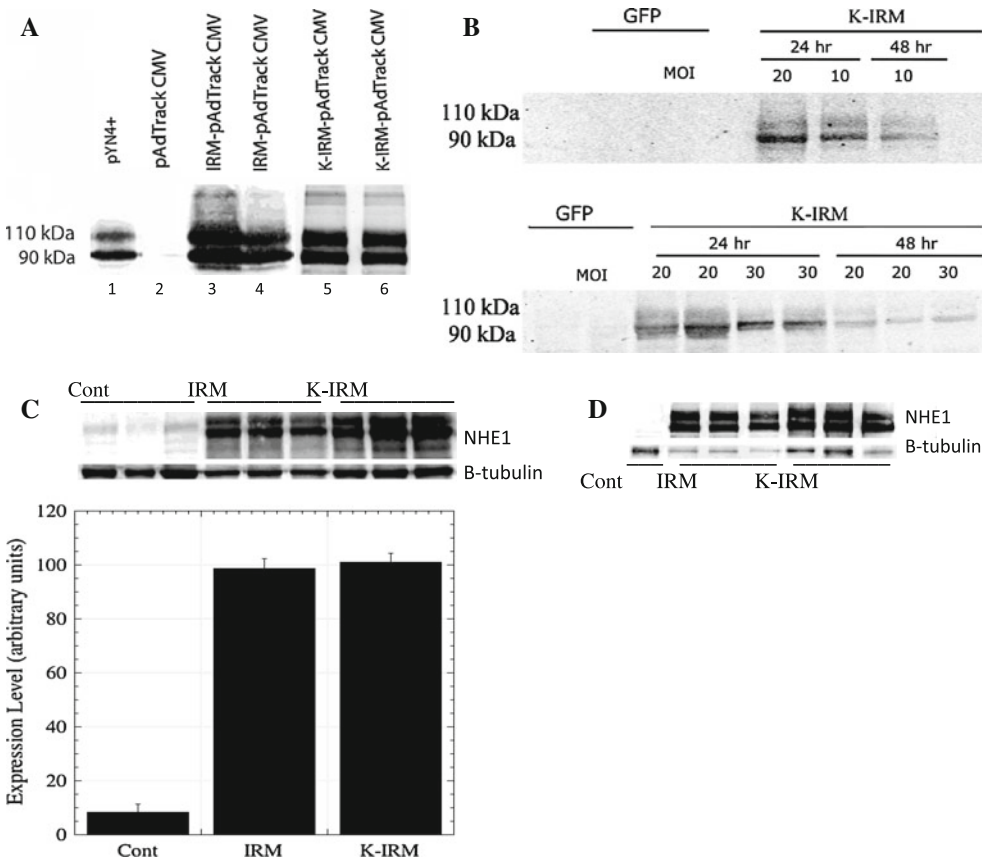

Fig. 2 Expression of NHE1 protein in AP-1 cells and in NRVM. **a** Western blot analysis of AP-1 cells transfected with pAdTrackCMV, pAdTrackCMV-IRM, or pAdTrackCMV-K-IRM plasmids. Representative western blot of AP-1 cells transfected with pAdTrack CMV-IRM or pAdTrack CMV-IRM K-IRM plasmids and immunoblotted with anti-HA tag antibody. *Lane 1* pYN4^{+}, is AP1 cells transfected with the pYN4^{+} plasmid, which contains an HA tagged-wild type NHE1 protein. *Lane 2* represents AP-1 cells transfected with pADTrack CMV, which contains no HA tag. *Lanes 3* and *4* represent lysates from AP-1 cells transfected with pAdTrack CMV-IRM, which contains the HA tagged-inhibitor resistant wild type NHE1. *Lanes 5* and *6* represent lysates from AP-1 cells transfected with pAdTrack CMV-K-IRM, which contains the HA tagged-inhibitor resistant active NHE1. **b** Multiplicity of infection (MOI) and time course of NHE1 protein expression in NRVM infected with the K-IRM adenovirus. NRVMs were infected with the K-IRM adenovirus (HA tagged-inhibitor resistant active NHE1) or GFP and the time course of expression was examined by western blotting against the anti-HA tag. Upper and bottom panel, representative western blots of NRVMs infected the K-IRM adenovirus using an MOI of 10, 20, or 30 of and examined at 24 and 48 h (h) post infection with anti-HA tag antibody. NRVMs were also infected with the GFP only containing adenovirus using an MOI of 20 for 24 h and blotted with anti-HA tag antibody. **c** Expression levels of total NHE1 protein in NRVM infected with either pAdTrackCMV, pAdTrackCMV-IRM, or pAdTrackCMV-K-IRM containing adenovirus. Immunoblotting was with anti-NHE1 antibody. *Upper panel* example of immunoblot. *Lower panel* quantification of relative levels of total NHE1 protein. **d** Western blot of expression of NHE1 protein in NRVM using anti-HA tag antibody. NRVMs were infected with a MOI of 20 of GFP (Cont), IRM (HA tagged-wild type NHE1), or K-IRM (HA tagged- actived NHE1) expressing adenoviruses for 24 h. NHE1 was detected by western blotting with a mouse monoclonal anti-HA (12CA5). *Upper panel* is a representative western blot of NHE1 detected at 90–110 kDa and lower panel is 50 kDa β-Tubulin, a loading control

physiological roles in cells. It acts as an anchor for other proteins including the actin cytoskeleton [47], so that increased NHE1 expression without increased activity, could therefore affect cardiac function.

We recently [29] examined the question of elevated NHE1 activity and protein levels in a transgenic mouse model. We demonstrated that expression of activated NHE1 caused significant increases in heart weight to body weight, and other indices of heart hypertrophy. However, in this kind of model it is still unclear as to whether the effects shown are direct effects of NHE1 on cardiomyocytes, or whether they are secondary, due to effects on heart development. NHE1 expression has been shown to affect cardiac stem cell development [48] and differentiation in various cell types [42]. Changes in cardiac development were shown in this study [29] so that it was unclear if effects on the heart were directly mediated by effects of NHE1 activity on cardiomyocytes cells, or indirectly through effects on cardiac development and differentiation in the myocardium.

In this study, we examined direct short-term effects of elevated expression of activated and wild type NHE1 on

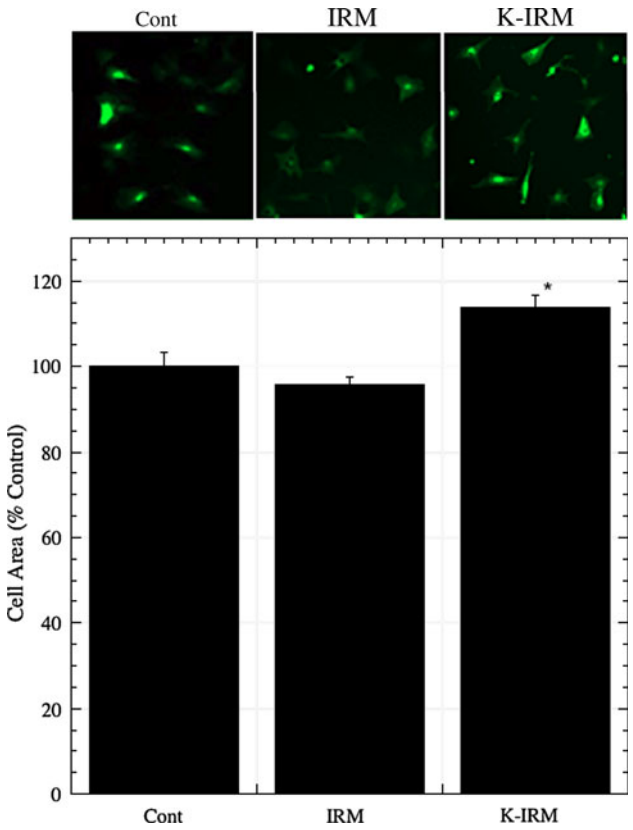

Fig. 3 Analysis of cell area of neonatal rat ventricular cardiomyocytes infected with IRM, K-IRM, or GFP expressing adenoviruses. NRVMs were infected with a MOI of 20 of GFP, IRM, or K-IRM adenoviruses for 24 h. *Upper panel* representative images of fluorescence microscopy of NRVMs infected with GFP, IRM, or adenoviruses. *Bottom panel* quantitative analysis of cell area. Results are expressed as %GFP ± %SEM. $^{*}P < 0.005$ for K-IRM vs. GFP, or IRM $n = 4$–7. At least 50 cells were measured from three individual dishes to represent one n value

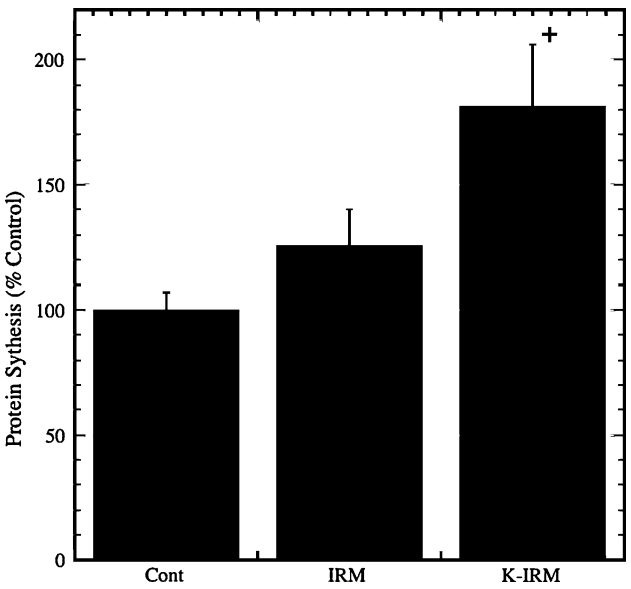

Fig. 4 Analysis of the rate of protein synthesis in neonatal rat ventricular cardiomyocytes infected with IRM, K-IRM, or GFP expressing adenoviruses. NRVMs were infected with a MOI of 20 of GFP, IRM, or K-IRM adenoviruses for 24 h. [^{3}H] Phenylalanine (1 μCi/ml) was added 4 h following adenoviral infection. Quantitative analysis of the rate of protein synthesis was measured by the amount of [^{3}H] phenylalanine incorporated. Protein synthesis was corrected with DNA content and was expressed as %GFP ± SEM. $^{\dagger}P < 0.005$ for K-IRM vs. GFP or IRM $n = 20$–30 plates, 5–8 experiments

isolated cardiac myocytes. Initial experiments confirmed that we could express significant levels of exogenous, HA-tagged NHE1 protein. The total levels of NHE1 protein in cells infected with wild type and activated NHE1 proteins were the same. However, we found different effects on cardiomyocytes. Increased expression of activated NHE1 protein resulted in significantly increased cardiomyocyte size and increased protein synthesis, while the wild type NHE1 protein did not have these effects. While the effects on size were not large, this occurred with a relatively short exposure to increased NHE1 levels. Longer times of exposure to the adenovirus resulted in reduced NHE1 expression levels (Fig. 2b) and thus were not used. Our measurement of cell size only recorded increases in two dimensions and effects on cell volume would be greater.

Earlier [36] we examined the effect of adenoviral infection of NHE1 on NHE1 activity. In assays which measured the maximal activity after transient acidosis, NHE1 activity was not significantly increased, though activity that was resistant to inhibitors made up approximately 50% of the active NHE1 protein. This indicated that the infected protein was expressed in active form in the cardiomyocytes. Preliminary experiments measuring NHE1 activity in this study also gave similar results (not shown). These results are in agreement with our recent observations [29] which showed that activation and regulation of NHE1 are critical to cause hypertrophy in the intact animal. Elevating the level of NHE1 alone was not strongly hypertrophic. Rather, it is the activation of the protein that is critical. The reason that we do not find large increases in the maximal rate of activity in the assays inducing acidosis, is not yet clear. However, it may be that the amount of NHE1 protein at the cell surface is limited. Presently it seems clear that the critical feature is not the total amount of cellular NHE1 protein, but rather the activation of the protein that is critical.

We also found that expression of activated NHE1 protein elevated protein synthesis. These effects were increased much more relative to changes in size. This indicated that enhanced NHE1 activity had greatly affected the protein biosynthetic pathway, an indication of a hypertrophic effect. We have recently demonstrated that expression of NHE1 in intact animal hearts, leads to expression of a variety of genes that are in the hypertrophic pathways [39]. Secreted phosphoprotein I (osteopontin)

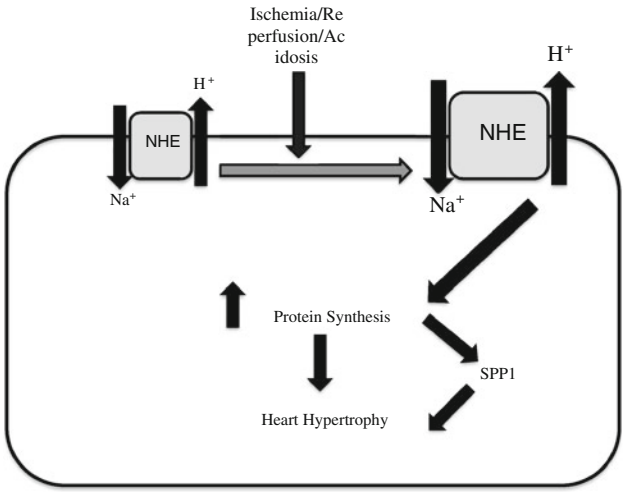

Fig. 5 Schematic diagram of hypertrophic pathway in cardiac myocytes mediated by the Na^+/H^+ exchanger. Illustration of putative pathways whereby elevation of NHE activity leads to heart hypertrophy in isolated cardiomyocytes. Ischemia/reperfusion or intracellular acidosis lead to increased NHE1 protein levels, and also increased NHE1 activity through stimulation of regulatory protein kinases [22]. Increased NHE1 activity stimulates protein synthesis and hypertrophic pathways, including elevation of secreted phosphoprotein 1 (SPP1) [39] which promotes a hypertrophic pathway

and its signaling pathways were up-regulated. They have been shown to be important in myocardial remodeling, cardiac hypertrophy and fibrosis [49–52]. Future experiments could involve examination of the direct effect of elevated NHE1 expression on secreted phosphoprotein I levels.

Previously, in an in vivo model of the effects of NHE1 expression [29] we examined other molecular pathways that may be involved in cardiac hypertrophy. Mitogen activated kinases such as ERK, p38, JNK, and RSK, all of which have been implicated in cardiac hypertrophy, were examined in transgenic mice expressing wild type NHE1 (N-line) or active NHE1 (K-line). We found no significant differences in the level of phosphorylated ERK, p38, JNK, RSK in hearts from N- and K-lines mice when compared to controls. It has been suggested that alterations of Ca^{2+} cycling mediate NHE1-induced cardiac hypertrophy. However, earlier [38], in vivo, we demonstrated that Ca^{2+} cycling (NCX, SERCA, and NKA) and pH regulatory proteins (AE3 and NBC1) were not altered in hearts from N- and K-lines mice. Calcineurin, a pro-hypertrophic serine–threonine protein phosphatase that is activated in response to sustained elevation of $[Ca^{2+}]_i$ was also investigated. Calcineurin's pro-hypertrophic effects have also been implicated in NHE1-induced cardiac hypertrophy. We also found that there were no differences in calcineurin activity in heart lysates from control, N- and K-lines mice [29]. These results suggested that activation of NHE1 causes myocardial hypertrophy through alternative pathways.

Overall our study has demonstrated that increased expression of activated NHE1, acts directly on isolated cardiac myocytes to promote a hypertrophic pathway (Fig. 5). Elevated expression of wild type NHE1 protein, was not enough to promote this pathway. We have shown [22] earlier that kinases that phosphorylate NHE1 have increased activity in the myocardium with ischemia and reperfusion. We have also demonstrated that sustained acidosis in the myocardium, promotes activation of NHE1 directed protein kinases [35]. We suggest that ischemic damage to the myocardium, with accompanying acidosis, activate the NHE1 protein which may itself be increased in amount. The elevated level of NHE1 activity leads to activation of a hypertrophic pathway, including elevation of secreted phosphoprotein I levels which we [39] and others [49–51, 53] have demonstrated earlier in various models of heart infarction or cardiac hypertrophy. This leads to promotion of hypertrophy directly in isolated cardiomyocytes (Fig. 5). Future experiments will explore this pathway further.

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