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Overexpression of the NHE1 isoform of the Na^+/H^+ exchanger causes elevated apoptosis in isolated cardiomyocytes after hypoxia/reoxygenation challenge

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Abstract The mammalian Na^+/H^+ exchanger isoform 1 (NHE1) is a ubiquitously expressed membrane protein that regulates intracellular pH in the myocardium and other tissues. NHE1 is an important mediator of myocardial damage that occurs after ischemia–reperfusion injury. It has also been implicated in apoptotic damage in many tissues and its expression and activity are elevated in disease states in the myocardium. In this study, we examined the effect of additional exogenous NHE1 expression on isolated cardiomyocytes susceptibility to ischemia/reperfusion damage. Exogenous NHE1 elevated Na^+/H^+ exchanger expression and activity when introduced into isolated cardiomyocytes through an adenoviral system. Isolated cardiomyocytes were subjected to simulated ischemia and reperfusion after infection with either control or NHE1-containing adenovirus. Cells were placed into an anaerobic chamber and effects of NHE1 expression after hypoxia/reoxygenation were examined. Hypoxia/reoxygenation increased caspase-3-like activity in controls, and the effect was greatly magnified in cells expressing NHE1 protein. It also elevated the percentage of apoptotic cardiomyocytes, which was also aggravated by expression of NHE1 protein. Hypoxia/reoxygenation also increased phospho-ERK levels. Elevated NHE1 expression was coincidental with increased expression of the ER stress protein, protein disulfide isomerase (PDI) and calreticulin (CRT). Our results demonstrate that increased NHE1 protein expression makes cells more susceptible to damage induced by hypoxia/reoxygenation in isolated cardiomyocytes. They suggest that elevated NHE1 in cardiovascular disease could predispose the human myocardium to enhanced apoptotic damage.

Keywords Apoptosis · Cardiomyocyte · Hypoxia · Intracellular pH · Ischemia–reperfusion · Na^+/H^+ exchanger

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Introduction

The Na^+/H^+ exchanger isoform-1 (NHE1) is a plasma membrane glycoprotein that protects cells from intracellular acidification by extruding one intracellular proton in exchange for one extracellular sodium [1]. Of the 10 known isoforms of Na^+/H^+ exchanger (NHE1–NHE10), NHE1 was the first isoform discovered [2] and is ubiquitously distributed. Other isoforms have more restricted tissue distributions, and some have predominantly intracellular localization. In mammals, aside from its role in pH regulation, NHE1 is also important in cell volume regulation, cell proliferation, and in metastasis of some tumor cells [1, 3]. In the myocardium, NHE1 plays several key roles in heart disease. It is critical in mediating the damage that occurs with ischemia/reperfusion of the myocardium [4, 5] and is an important mediator of heart hypertrophy [6]. Clinical trials are testing NHE1 inhibitors for treatment of various forms of heart disease [7].

The NHE1 isoform of the Na^+/H^+ exchanger was identified as the predominant isoform in the myocardium [8], where it is concentrated along the intercalated discs and transverse tubule system [9]. In addition to its pH regulatory role, NHE1 is also important in apoptosis in the myocardium. Apoptosis significantly contributes to myocyte loss following myocardial infarction [10–14], and many detrimental effects of NHE1 in hypoxia–reoxygenation are mediated or

compounded by apoptosis [15–20]. Inhibitors of NHE1 activity decrease apoptosis in isolated cardiomyocytes [21] and in fibroblasts [18]. NHE inhibition before ischemia reduces myocardial apoptosis in isolated rat hearts [19, 20], in intact rats [22, 23], in mouse hearts [23], and in pacing-induced heart failure in rabbits [24]. Chronic NHE1 blockade has an antiapoptotic effect in the hypertrophied heart [25] as does inactivation of the NHE1 gene following transient cerebral ischemia [16]. Regulation of NHE1 has been implicated in NHE1 induced apoptosis in the myocardium [26, 27] and in other tissues [28, 29].

It is of note that NHE1 levels and activity are elevated in several models of heart disease. There is increased NHE1 activity in hypertensive, hypertrophied, or diabetic myocardium [30]. NHE1 activity is also elevated in human patients with end stage heart failure [31], and activity and mRNA levels are elevated in isolated cardiomyocytes subjected to chronic acidosis [32, 33]. In addition, we demonstrated that protein kinases that regulate NHE1 activity are activated in the myocardium by ischemia and reperfusion of intact hearts or isolated cardiomyocytes [34]. The elevation of NHE1 activity in the myocardium in myocardial disease raises questions regarding whether increased NHE1 activity is maladaptive to the myocardium. In this study, we examined the effect of NHE1 overexpression on the induction of apoptosis in isolated cardiomyocytes. We found that elevation of NHE1 levels had a pro-apoptotic effect in isolated cardiomyocytes subjected to ischemia followed by reperfusion. The results suggest that increased NHE1 levels in the mammalian myocardium may make the heart more amenable to apoptotic damage induced by ischemia/reperfusion.

Materials and methods

Materials

Routine chemicals were of analytical grade and were purchased from Sigma (St. Louis, MO, USA), Fisher Scientific (Ottawa, ON), or BDH (Toronto, ON). Special chemicals were collagenase Type 2 obtained from Worthington Biochemical Corporation (Lakewood, NJ) and BCECF-AM (2′,7-bis(2-carboxyethyl)-5(6) carboxyfluorescein-AM) from Molecular Probes (Eugene, OR, USA). Also, platinum R Taq Polymerase was from Invitrogen (Burlington, Ont.) and EMD87580 was a generous gift of Dr. N. Beier of Merck KGaA, Frankfurt, Germany. PWO DNA polymerase was obtained from Roche Applied Science and Lipofectamine™ 2000 reagent was from Invitrogen. Dithiothreitol (DTT) and 3,4,5-dimethyl thiazol-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA), and N-Acetyl-Asp-Glu-Val-Asp-p-nitroaniline (Ac-DEVD-pNA) was from Alexis Biochemicals (Lausen, Switzerland). Mouse anti-NHE1 antibody was from BD Biosciences (San Jose, CA, USA). Phospho-ERK1/2 (Thr202/Tyr204)- Mouse Monoclonal antibody and ERK1/2 (p44/42 MAPK)- Rabbit polyclonal antibody were from Cell Signaling Technology (Danvers, MA, USA). Goat polyclonal anti-CRT antibody and rabbit polyclonal anti-PDI antibody were a generous gift of Dr. M. Michalak, Dept. of Biochemistry, University of Alberta.

Preparation and treatment of isolated cardiomyocytes

Primary cultures of cardiomyocytes were prepared from 5 to 6-day-old neonatal Sprague–Dawley rat heart ventricles as described earlier [35]. Isolated primary cardiomyocytes were plated onto glass coverslips for intracellular pH measurements, or onto Corning culture dishes or flasks when harvesting cell extracts. Myocytes were maintained for 48 hours prior to adenoviral infection 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.

Where indicated, isolated cardiomyocytes were infected with adenovirus containing HA-tagged NHE1 gene. The adenoviral construct (pADTRack-CMV-NHE1) was described earlier [36] and contains full-length cDNA of the NHE1 isoform of the Na^+/H^+ exchanger. The NHE1 isoform expressed contains a double mutation Leu163Phe/Gly174Ser that increases the resistance to inhibition by EMD87580 and other NHE1 inhibitors [37]. This allows detection of its activity while inhibiting activity of the endogenous NHE1 protein. Infection by adenovirus was monitored by GFP expression and control cells were infected with an adenovirus that expressed GFP alone. Cells were routinely infected at a multiplicity of infection of 20. Control or experimental adenovirus were maintained on cells for a period of 24 h prior to treatments.

Isolated cardiomyocytes were treated with simulated ischemia and reperfusion after infection with either control or NHE1-containing adenovirus. Cells were placed into an anaerobic chamber (GasPac system, BD Biosciences) at 37°C for 14 h as described earlier [38]. The chamber contained a disposable hydrogen and carbon dioxide generator envelope and an oxygen-consuming palladium catalyst. On activation, it created a hypoxic condition of 25–35 mmHg PO_2 [38] and the anoxic condition inside the chamber was ensured with a methylene blue anaerobic indicator. After hypoxia, the chamber was opened and cells

were placed in a standard incubator (95% air, 5% CO_2) for 2 h for reoxygenation prior to assay. Some experiments were in the presence of 10 μM EMD8750, which was added 20 min prior to hypoxia and maintained throughout the reoxygenation period.

Intracellular pH measurement

In order to examine NHE1 activity in isolated cardiomyocytes, cells were mock infected, infected with pAd-NHE1, or pAd-GFP, a control adenovirus expressing only GFP as described above. Cells were infected for approximately 24 h prior to intracellular pH measurement [39]. A population of isolated cardiomyocytes grown on coverslips was measured using a PTI Deltascan spectrofluorometer. The initial rate of Na^+-induced recovery of cytosolic pH (pH_i) was measured after an ammonium chloride pulse (50 mM × 3 min) induced an acute acid load. BCECF-AM was used for fluorescence measurements, and recovery was measured in the presence of 135 mM NaCl as described previously [40]. The initial rate of recovery was measured during the first 20 s after return of NaCl at 37°C. In order to test the efficacy of inhibitors of NHE1 and to determine the presence of exogenous and active NHE1, we used a two pulse assay in which the second pulse was done in the presence of 10 μM EMD87580 [39]. Cells are treated with ammonium chloride two times and allowed to recover in NaCl-containing medium. For the first treatment with ammonium chloride, cells are rinsed with Na^+-free medium for 10–20 s and when the decline in pH_i has stabilized, NaCl is added immediately to allow for intracellular pH recovery. The second pulse was done under the same conditions except that the recovery was in the presence of 10 μM EMD87580 [39]. EMD87580 was dissolved in PBS and added during ammonium chloride treatment, in Na^+-free medium, and during recovery in NaCl. We previously [36] showed that 10 μM EMD87580 was sufficient to inhibit endogenous NHE1 in isolated cardiomyocytes, but it does not inhibit exogenous NHE1 with the Leu163Phe/Gly174Ser mutation. A calibration curve was done with nigericin at the end of every experiment to calibrate intracellular pH to fluorescence as described earlier [40]. Results are shown as the mean ± SE.

Buffering capacity (B, mmol/liter/pH unit) was determined essentially as described earlier [41] by varying the amount of NH_4Cl and by the observing the change of intracellular pH produced by this load. Isolated cardiomyocytes that were infected with various adenoviruses were incubated with NH_4-containing buffer as described earlier [41].

$$\text{buffering capacity}(B) = \Delta NH_4 + i/\Delta pH_i$$
$$(NH_4Cl_o \times 10(pK_a - pH_i))/[1 + 10(pK_a - pH_o)]$$

The Henderson–Hasselbach relation using a pK_a for NH_4^+ of 9.21 determined the equilibrium between NH_4^+, NH_3, and pH in the extracellular medium.

Caspase-3-activity

Caspase-3-like activity assays were performed to detect apoptotic cell death as we have described earlier [42]. In order to assay caspase-3-like activity cells were harvested and washed once with ice-cold phosphate buffered saline. After washing, 100 μl of cell lysis buffer (20 mM HEPES–NaOH, pH 7.5, 100 mM NaCl, 0.25% Triton-X, 1 mM EDTA, 1 mM DTT, 1 mM phenylmethanesulfonylchloride, 10 μg/ml leupeptin) was added, and cells were incubated on ice for 10 min. The lysates were collected, and after centrifugation at 14,000 × g for 10 min, the supernatant was collected, and protein concentration was determined. The reaction was carried out in 96-well plates and started by adding equal amounts of proteins (20 μg) in caspase assay buffer (20 mM HEPES–NaOH, pH 7.0, 100 mM NaCl, 1 mM EDTA, 10% glycerol and 10 mM DTT) with 50 μM Ac-DEVD-pNA. After 1 h incubation at 37°C, the caspase activity was measured by monitoring the release of pNA at 405 nm in plate reader (Molecular Devices, CA, USA). Results are mean ± SE and statistical significance was determined using a Mann–Whitney *U* test or a Students *t* test.

Hoechst staining

Cells grown on coverslips were fixed in 4% paraformaldehyde for 20 min at room temperature and then treated with 5 μg/ml Hoechst 33258 (Sigma) for 20 min at 37°C in the dark. After brief rinsing with phosphate buffered saline (PBS), the cells were observed under fluorescence microscope. At least, 1,000 cells were counted from 10 randomly selected microscope fields for each group.

Western blotting

SDS–PAGE and immunoblotting were performed essentially as described earlier [43]. For Western blot analysis, equal amounts of up to 100 μg of each sample were resolved on 10% SDS/polyacrylamide gels. Nitrocellulose transfers were immunostained using anti-HA monoclonal antibody for NHE1 detection (Boehringer Mannheim, Laval, Que., Canada) or anti-NHE1 monoclonal antibody (BD Biosciences). Second antibody was peroxidase-conjugated goat anti-mouse antibody (Bio/Can, Mississauga, ON, Canada) and visualized using a chemiluminescence detection system. MF 20 anti-myosin antibody was from the Developmental Studies Hybridoma Bank, University of

Iowa, Iowa City, IA, USA. For all other blots to visualize and quantify immunoreactive proteins, we used a Li-COR fluorescence labeling and detection systems (LI-COR Biosciences, Lincoln, Nebraska USA). In order to detect ERK1/2 and phosphoERK1/2, the two antibodies were used together at 1:1000 dilution, and then visualized with the appropriate secondary antibodies.

Results

In order to increase the level of NHE1 protein in isolated cardiomyocytes, we used an adenoviral vector which we have described earlier [36]. We initially confirmed that the adenoviral vector was expressing the HA-tagged NHE1 protein. The adenovirus used also co-expresses GFP with NHE1. Figure 1a illustrates the results. Infection of the cells with adenoviral vector caused over 99% infection of primary cultures of isolated cardiomyocytes. Uninfected cells showed no fluorescence (not shown).

Western blot analysis with anti-HA antibodies was used to confirm specific NHE1 expression in cardiomyocytes. Infection with Ad-NHE1 which contains the HA-tagged adenovirus (Fig. 1b, lane 2, top panel) demonstrated NHE1-HA expression in primary cultures of neonatal cardiomyocytes when infected with adenovirus expressing NHE1. Infection of cells with adenovirus expressing only GFP protein did not result in any NHE1 detectable with anti-HA antibodies (Fig. 1b, lane 1, top panel). A Western blot of the same samples probed with anti-myosin antibody demonstrated that approximately equal amounts of sample were present (lower panel). These results demonstrated that the HA-tagged NHE1 protein was expressed in cardiomyocytes that were infected with the Ad-NHE1 adenovirus. Infection with adenovirus that did not contain NHE1-HA protein confirmed that the expression was specific to the HA-tagged NHE1 protein insert in the Ad-NHE1 adenovirus.

In order to examine the level of expression of NHE1 in isolated cardiomyocytes, we used an antibody against the NHE1 protein itself. Figure 1c illustrates the results. Lane 1 illustrates a positive control made from CHO cells stably transfected with NHE1 protein. It demonstrated strong immunoreactivity with NHE1 protein. Lane 2 is cell extract from mock-infected isolated cardiomyocytes which demonstrated immunoreactivity of the endogenous rat NHE1 protein. Lanes 3 and 4 are cell extracts infected with adenovirus that does not express NHE1 (Lane 3, Ad-GFP adenovirus) or that which expresses NHE1 (Lane 4, Ad-NHE1). Cells infected with adenovirus expressing only GFP show a level of endogenous NHE1 protein comparable to that of mock-infected cardiomyocytes. Cells infected with Ad-NHE1 adenovirus which expressed NHE1 showed an increased level of expression of NHE1 protein which was likely due to both the endogenous NHE1 plus the exogenously expressed NHE1 from the adenovirus. All the samples expressed NHE1 protein of approximately 110 kDa in size, which represents the fully glycosylated NHE1 protein. A slightly smaller partially or de-glycosylated NHE1 protein is also evident, which is typical of results seen earlier [43].

Figure 1d and e show an example and a summary of the results illustrating the activity of NHE1 as measured in a dual pulse assay. There were no differences in the buffering capacity of cells infected with control or NHE1 expressing adenovirus. There was no difference in the level of acidification induced by ammonium chloride in infected cells. The absolute level of activity of mock-infected cells in the first pulse was 0.31 ± 0.04 ΔpH/min. This was unchanged in GFP-infected cells but significantly increased to 0.36 ± 0.04 in the AdNHE1-infected cells ($P < 0.05$). In order to determine whether we had successfully expressed active exogenous NHE1 protein, we used a two pulse assay in which the second pulse was ± the NHE1 inhibitor EMD87580. In the absence of EMD87580, the second pulse was 86–88% of the first pulse in all the cases (Fig. 1d, e). For both mock- and GFP-infected cells, EMD87580 eliminated over 95% of this recovery (Fig. 1e, lanes ME and GE). For NHE1 cells infected with the inhibitor resistant NHE1 protein, approximately half the NHE1 activity remained (Fig. 1e, lane NE). The decrease in NHE1 activity obtained was presumably due to inhibition of endogenous NHE1, while the remaining NHE1 activity was likely due to the exogenous, inhibitor-resistant NHE1.

The activation of caspases is a major biochemical marker of apoptotic cell death and various studies have already shown this event as a prerequisite for induction of apoptosis in cardiomyocytes [44]. Therefore, we initially tested the effect of inhibition of NHE1 on the caspase-3-like activity that was induced by ischemia/reperfusion-like treatment of isolated cardiomyocytes. The results are shown in Fig. 2. Hypoxia alone induced approximately a 3-fold increase in caspase-3-like activity while hypoxia followed by reperfusion further increased activity significantly greater than hypoxia alone. Treating the cells with EMD87580 prevents the increase in caspase-3-like activity, reducing the activity to a level that was not significantly different from the controls.

We further characterized the effects of hypoxia/reoxygenation and EMD87580 treatment on isolated cardiomyocytes by examining the % of apoptotic cells. Cells were treated with hypoxia/reoxygenation ± EMD87580 as described above and the percentage of apoptotic cells was estimated by staining with Hoechst as described in the "Materials and methods". Cells with fragmented or

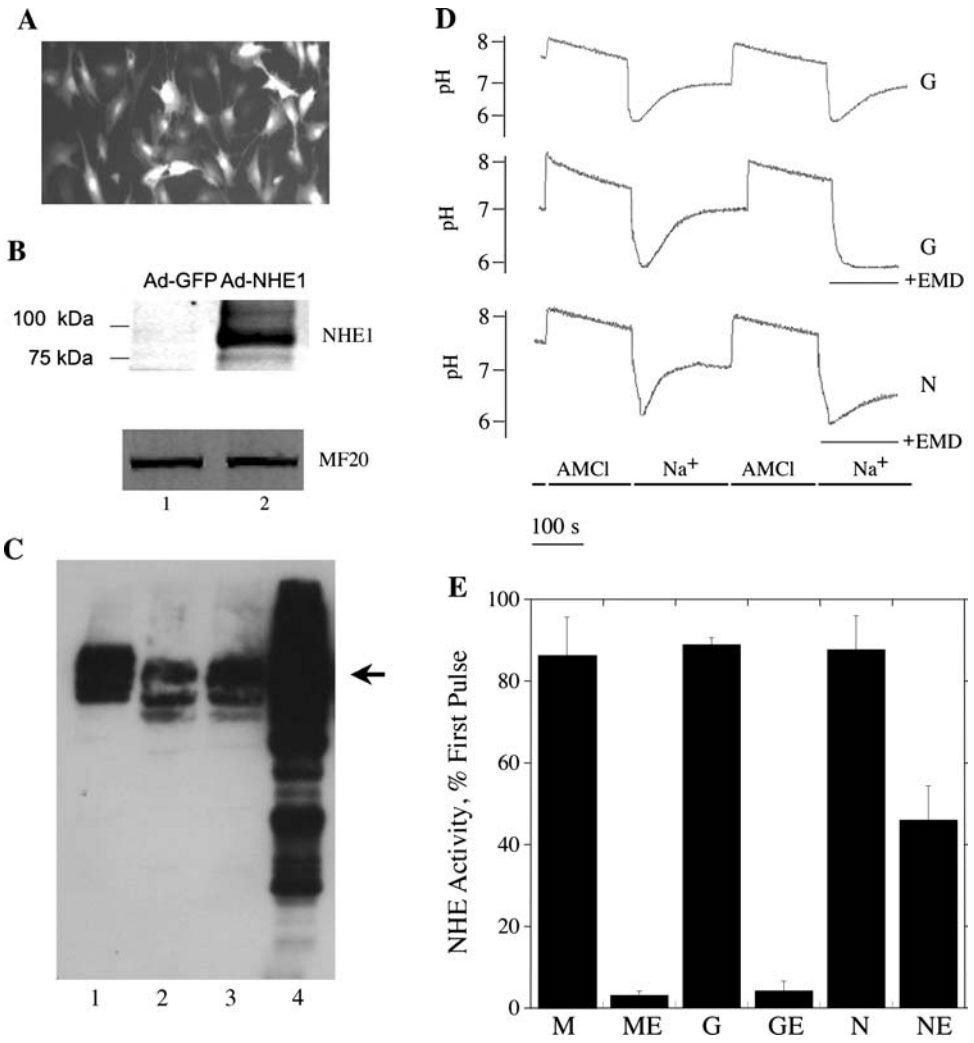

Fig. 1 Expression of exogenous Na^+/H^+ exchanger in isolated cardiomyocytes infected with adenovirus containing NHE1 protein (pAdNHE1). Isolated neonatal cardiomyocytes were made from rat hearts as described in the "Materials and methods". Cells were then infected with pAdNHE1 containing the HA-tagged inhibitor-resistant NHE1 protein. **a** GFP reporter fluorescence of isolated cardiomyocytes infected with pAdNHE1. **b** Western blots of cell extracts were blotted with anti-HA antibody. Lane 1 was from cells infected with a control adenovirus (Ad GFP) which had no NHE1. Lanes 2 was an extract from isolated cardiomyocytes infected with pAdNHE1 which contains the HA-tagged inhibitor resistant NHE1 isoform of the Na^+/H^+ exchanger. Lower Panel, Western blot of lanes 1–2 of re-probed with antibody to myosin (MF-20). **c** Western blots of cell extracts were blotted with anti-NHE1 antibody. Lane 1 was a control from AP-1 cells stably infected a plasmid containing HA-tagged NHE1. Lane 2 is a cell extract from mock-infected isolated cardiomyocytes. Lane 3 is a cell extract from isolated cardiomyocytes with a control adenovirus (Ad GFP) which had no NHE1. Lanes 4 was an extract from isolated cardiomyocytes infected with pAdNHE1 which contains the HA-tagged inhibitor resistant NHE1 isoform of the Na^+/H^+ exchanger. The arrow denotes the fully glycosylated NHE1 protein. **d** Representative tracings of typical assay measuring the rate of recovery from an acid load in isolated cardiomyocytes. Acid load was induced using 50 mM ammonium chloride (AMCl) for 3 min followed by a brief incubation in Na^+-free buffer. The rate of recovery from an acid load was measured for 3 min in Na^+ normal containing buffer (Na^+). The ammonium chloride treatment and recovery were repeated and 10 μM EMD87580 was added where indicated. Intracellular pH was monitored using BCECF as indicated in the "Materials and methods". "G" indicates cells were infected with adenovirus expressing GFP protein. "N" indicates cells were infected with adenovirus expressing NHE1. **e** Summary of results measuring activity of NHE1 protein in the presence or absence of EMD87580. Cells were subjected to two pulses of ammonium chloride with two recovery periods. The value of the rate of recovery of the second pulse was compared to that of the first pulse. Where indicated, the second pulse contained 10 μM EMD87580 (E). "M" indicates mock-infected cells. "G" and "N" indicate cells were infected with adenovirus expressing GFP and NHE1, respectively. "ME" cells mock infected and assayed with the addition of 10 μM EMD87580 present during the second pulse of the assay. "GE" cells infected with adenovirus for GFP and assayed with the addition of 10 μM EMD87580 present during the second pulse of the assay. "NE" cells infected with adenovirus for inhibitor-resistant NHE1 and assayed with the addition of 10 μM EMD87580 present during the second pulse of the assay

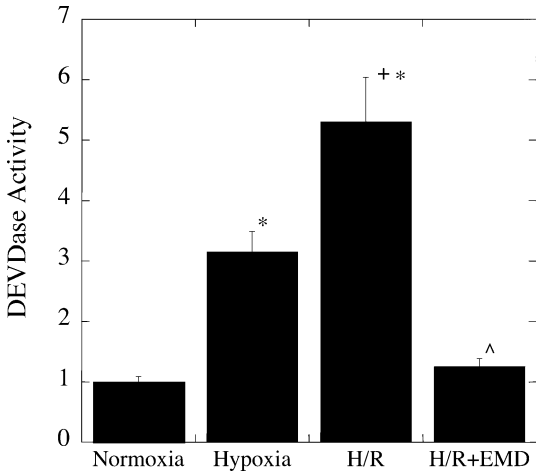

Fig. 2 Effect of EMD87580 on caspase-3-like activity of isolated cardiomyocytes subjected to hypoxia/reoxygenation. Isolated cardiomyocytes were prepared as described in the "Materials and methods" and were treated with hypoxia (14 h) or hypoxia followed by reperfusion (2 h, H/R) ± 10 μM EMD8750. Caspase-3-like activity was assayed as described in the "Materials and methods". Results are the mean ± SE of 9 determinations. * Significantly different from control (normoxia) cells at $P < 0.01$. $^{+}$ Significantly different from hypoxia treated cells at $P < 0.01$ and $^{\wedge}$ significantly different from H/R treated cells at $P < 0.01$

condensed nuclei were scored as apoptotic cells. The results are shown in Fig. 3a, b. Treatment of cells with hypoxia/reoxygenation increased the percentage of apoptotic cells approximately fivefold over basal levels. EMD87580 significantly reduced, but did not eliminate, the effects of hypoxia/reoxygenation. We then examined the effects of expression of additional NHE1 on the % of apoptotic cells after treatment with hypoxia/reoxygenation. The results are shown in Fig. 3c, d. A control infection was with adenovirus expressing only GFP protein. When these cells were treated with hypoxia/reoxygenation, the % of apoptotic cells increased significantly from approximately 5–26%. When cells were infected with adenovirus expressing NHE1, in normoxic conditions, the % of apoptotic cells rose significantly to almost 9%. Treatment with hypoxia/reoxygenation increased this significantly to almost 40%, and this was also significantly elevated in comparison to cells treated with control adenovirus (Fig. 3d).

In order to further confirm the effects of elevated expression of NHE1 on susceptibility to hypoxia/reoxygenation, we again used the adenoviral system to overexpress NHE1 in isolated cardiomyocytes and examined another parameter, caspase-3-like activity. We examined cells that were overexpressing NHE1, versus. a control adenoviral infection of GFP. The results are shown in Fig. 4. Expression of NHE1 alone increased the resultant caspase-3-like activity. Hypoxia/reoxygenation increased caspase-3-like activity in uninfected cells and in GFP-infected cells, but the effect was greatly increased in cells expressing NHE1 protein. As the NHE1 protein that was infected was resistant to inhibition by EMD87580, it did not reduce caspase-3-like activity in NHE1-infected cells. However, in GFP-expressing cells, the endogenous NHE1 was apparently inhibited by EMD87580 and caspase-3-like activity was reduced by EMD87580.

In another series of experiments, we examined kinases activated by hypoxia and reperfusion. Cell extracts from cardiomyocytes treated with hypoxia followed by reperfusion showed elevated levels of phospho-ERK confirming that this pathway is activated with the treatments given, and being consistent with the effects that we and others have seen earlier [34] (Fig. 5). Hypoxia alone did not result in elevation of phospho-ERK levels (Fig. 5a). We found that levels of both phospho-ERK1 (upper band) and phospho-ERK2 (lower band) were increased by hypoxia and reperfusion (Fig. 5a). There appeared to be more ERK1 protein present relative to the level of ERK2 (Fig. 5b), though it is unclear whether this is due to differences in efficacy of the antibody toward these proteins.

It has earlier been reported that the elevation of NHE1 levels in a transgenic model results in increased expression of ER stress proteins [45]. In order to examine whether ER stress proteins were elevated, we used Western blotting against two ER stress proteins, CRT and PDI. Western blotting of cardiomyocyte extracts with antibodies against CRT demonstrated a significant increase in CRT expression with adenoviral expression of NHE1 (Fig. 6a, b). In addition, expression of PDI was affected. Adenoviral expression of NHE1 increased PDI levels significantly compared to the control adenoviral infection (Fig. 6c, d).

Discussion

The NHE1 isoform of the Na^+/H^+ exchanger is the key pH regulator of the isolated cardiomyocyte and is involved in indirectly mediating both ischemia reperfusion damage to the myocardium and heart hypertrophy (See [46] for review). NHE1 levels and activity have been shown to be elevated with several types of cardiovascular stress [30–33]. We, therefore, examined the effect of elevation of NHE1 on the sensitivity of isolated cardiomyocytes to ischemia/reperfusion damage inducing apoptosis. We have earlier developed a system whereby we can express NHE1 in isolated cardiomyocytes and inhibit endogenous NHE1 [36, 43]. In this study, we used the same system to examine the effects of elevation of NHE1 expression on the susceptibility to ischemia/reperfusion damage. It should be noted that NHE1 is highly conserved between mammalian species. The N-terminal membrane domain of NHE1 contains an initial more variable first transmembrane segment and extracellular loop involved in targeting, but the

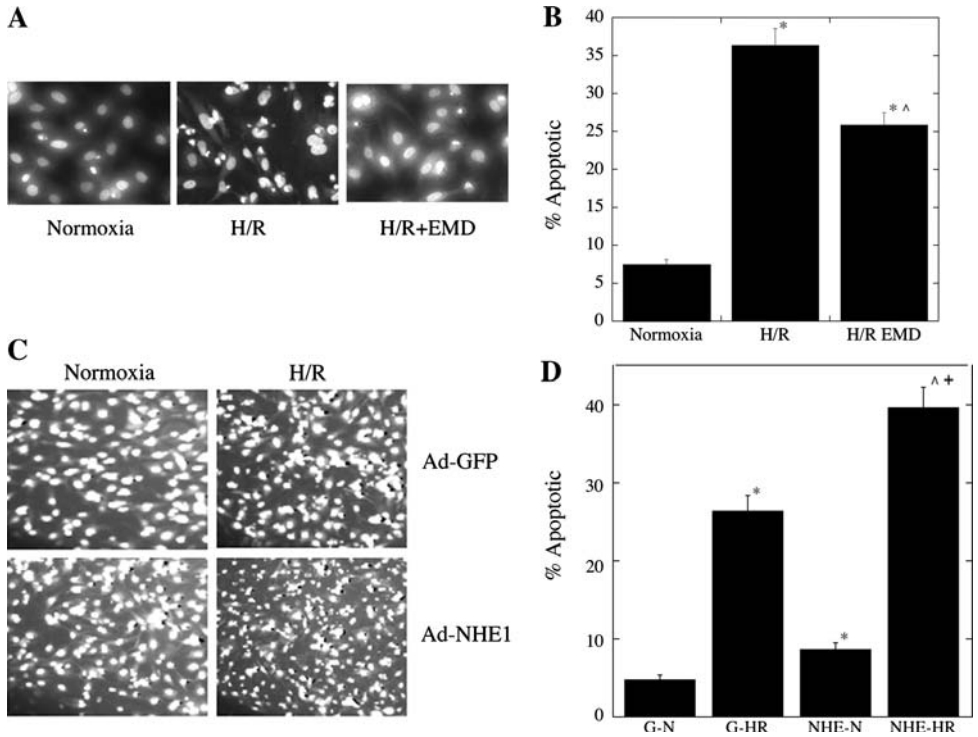

Fig. 3 Effects of EMD87580 on the percentage of apoptotic-isolated cardiomyocytes after hypoxia/reoxygenation. Isolated cardiomyocytes were prepared and treated as for Fig. 2. Apoptotic cells were estimated by staining with Hoechst as described in the "Materials and methods". **a** and **b**, Cells were not treated with adenovirus. **a** Example of staining of normoxic and hypoxia/reoxygenation (H/R) ± EMD87580 (EMD). **b** Summary of results estimating percentage of apoptotic cells. Results are the mean ± SE of 6 different experiments with a total of over 1,000 cells counted in each group. * Significantly different from control (normoxia) cells at $P < 0.01$. ^ Significantly different from H/R treated cells at $P < 0.01$. **c** and **d**, cells were treated with either adenovirus expressing GFP (Ad-GFP) or adenovirus expressing NHE1 protein (Ad-NHE1). **c** Examples of staining of normoxic and hypoxia/reoxygenation (H/R) cells treated with either control adenovirus (Ad-GFP) or adenovirus expressing NHE1 protein (Ad-NHE1). **d** Summary of results estimating percentage of apoptotic cells. G and N, cells treated with GFP-expressing adenovirus and normoxia. G-HR, cells treated with GFP-expressing adenovirus and hypoxia/reoxygenation. NHE-N, cells treated with NHE expressing adenovirus and normoxia. NHE-HR cells treated with NHE-expressing adenovirus and hypoxia/reoxygenation. * Significantly different from G and N cells at $P < 0.01$. ^ Significantly different from NHE-N cells at $P < 0.01$. + Significantly different from G-HR cells at $P < 0.01$

balance of the human and rat membrane domain is 95% identical in sequence [47]. The C-terminal cytosolic amino acids are slightly more divergent [47] but still retain over 92% identity and a higher amount of similarity in amino acids.

When cells were infected with adenovirus containing exogenous NHE1, we found that we had elevated NHE1 dramatically. Immunoblotting with an antibody against the HA tag confirmed that we had expressed exogenous protein (Fig. 1b). A different antibody against the NHE1 protein itself demonstrated that the total NHE1 level was significantly increased (Fig. 1c). The exogenous NHE1 was from a human cDNA while the endogenous protein was a rat cardiomyocyte protein. Cells infected with exogenous NHE1 appeared to contain a much larger amount of the protein; however, we did not know whether the immunoreactivity is precisely the same in the two species, so that a direct comparison of NHE1 levels is not quantitative. Nevertheless, it is clear that the amount of NHE1 protein is increased. The activity of the NHE1 protein was also elevated significantly, though not by a large amount. This is somewhat surprising considering that much more NHE1 protein appears to have been expressed. It may be that there are limits on the amount of functional NHE1 protein at the specific location of NHE1 in the plasma membrane. Perhaps, these are imposed by regulatory cofactors, or by the amount of regulatory modification of the protein. In our study, it was clear that we had increased the amount of NHE1, and the inhibition of endogenous NHE1 by EMD87580 showed that about half the active NHE1 protein present was exogenous EMD87580-resistant protein, while about half was endogenous protein.

Hypoxia followed by reperfusion elevated the level of caspase-3-like activity in isolated cardiomyocytes. In cells that express additional NHE1, the effect was even more pronounced. This confirmed our basic hypothesis that elevation of NHE1 levels caused increased sensitivity to apoptosis, induced by hypoxia/reoxygenation. Infection

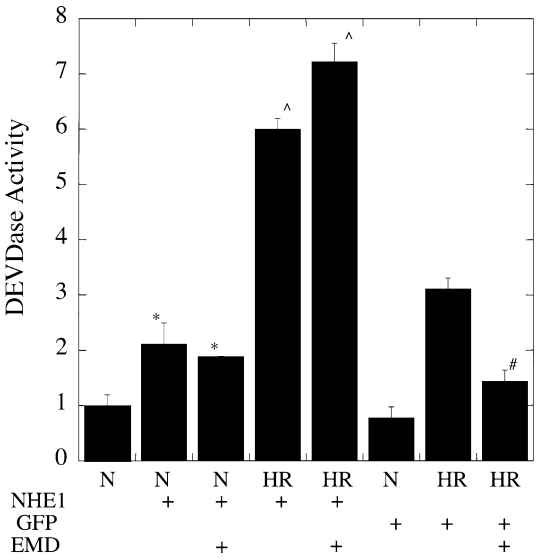

Fig. 4 Effect of expression of exogenous NHE1 on caspase-3-like activity of isolated cardiomyocytes subjected to hypoxia/reoxygenation. Isolated cardiomyocytes were prepared and untreated (normoxia, N) or hypoxia/reoxygenation (HR) as described in Fig. 2 in the presence or absence of 10 μM EMD87580. Cells were infected with adenoviral vectors expressing either NHE1 or GFP where indicated. Caspase-3-like activity was assayed. * Significantly different from corresponding uninfected cells at $P < 0.01$. ^ Significantly different from corresponding GFP-infected cells at $P < 0.01$. # Significantly different from cells not treated with EMD87580

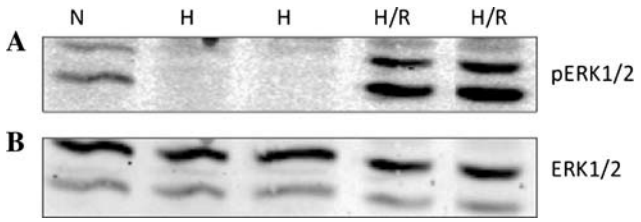

Fig. 5 Western blot analysis of ERK and Phospho-ERK levels in cells treated with hypoxia (H) or hypoxia followed by reoxygenation (HR). **a** Immunoblotted with anti-phospho-ERK antibody showing phosphorylated ERK1 and ERK2 protein. **b** Immunoblotted with anti-ERK protein antibody showing total ERK protein 1 and 2

with adenovirus alone, which did not express NHE1, did not have this effect. Confirmation of the enhanced sensitivity of the cells to hypoxia/reoxygenation damage was obtained in experiments in which we examined the % of apoptotic cells. Expression of exogenous NHE1 made the cells more sensitive to hypoxia/reoxygenation damage and increased the % of affected cells significantly.

The mechanism by which elevated NHE1 expression increases the susceptibility to hypoxia/reoxygenation is uncertain at this time. NHE1 activity was elevated, but not greatly. Treatment of NHE1-infected cardiomyocytes with EMD87580 did not reduce this effect. EMD87580 inhibited about half the activity of NHE1-infected cells, with the balance being due to EMD-resistant NHE1 from the adenovirus. This would be expected to reduce susceptibility to hypoxia/reoxygenation, but it did not. It may be due to that the elevation of NHE1 levels increases cardiomyocyte hypoxia/reoxygenation damage through other mechanisms. NHE1 has many other cellular functions aside from pH regulation including binding to the cytoskeleton, and association with other proteins [48, 49]. It may be that one of these other functions is involved. It has also recently been shown that elevation of NHE1 levels in the myocardium causes increased levels of some ER stress proteins [45]. We did indeed find that the ER stress proteins, PDI and CRT, were significantly elevated by the expression of NHE1 in isolated cardiomyocytes. Intentional induction of chronic ER stress has been shown to enhance apoptosis in cardiomyocytes [50], and Cook et al. [45] have suggested that there is a biphasic response to ER stress in the mouse myocardium. There may initially be an adaptive and beneficial response. However, unresolved ER stress may facilitate cardiac remodeling and failure, possibly through apoptosis and through defects in protein quality control. Further experiments are, therefore, necessary to confirm whether the mechanism of sensitization of the myocardium to hypoxia/reperfusion damage in our model, involves the latter maladaptive component of the ER stress response.

Our results confirmed that hypoxia followed by reoxygenation activated ERK1/2. We have earlier noted this observation [34]. Activation of ERK1/2 has been shown to be a key upstream signaling pathway responsible for doxorubicin-induced cardiomyopathy [51]. In addition, ERK activation has been shown to be important in other models which induce apoptosis in the myocardium including isoproterenol-induced apoptosis [52], and another similar model of ischemia/reperfusion in neonatal cardiomyocytes that induces apoptosis [53]. However, it is not clear whether NHE1 plays a role in mediating apoptosis in these models.

We did find that inhibition of NHE1 activity reduced the sensitivity to hypoxia/reoxygenation damage in cells without exogenous NHE1. There was a reduction, but not elimination, of the effects of hypoxia reperfusion on apoptosis. It has been reported earlier that NHE1 is involved in induction of cardiomyocyte apoptosis [17, 21], and chronic inhibition of NHE1 has recently been shown to decrease apoptosis in the myocardium [20, 25]. In other tissues, inhibition of NHE1 has also been reported to be associated with anti-apoptotic effects [54]. Our results confirm this effect in our system. Inhibition of endogenous NHE1 with EMD87580 reduced the damage induced by hypoxia/reoxygenation in isolated cardiomyocytes.

Our study is the first that directly demonstrates that elevated NHE1 expression directly enhances the apoptotic effects of NHE1. The results suggested that the elevation of NHE1 levels that occurs could predispose the myocardium to more damage through apoptosis. Future studies will

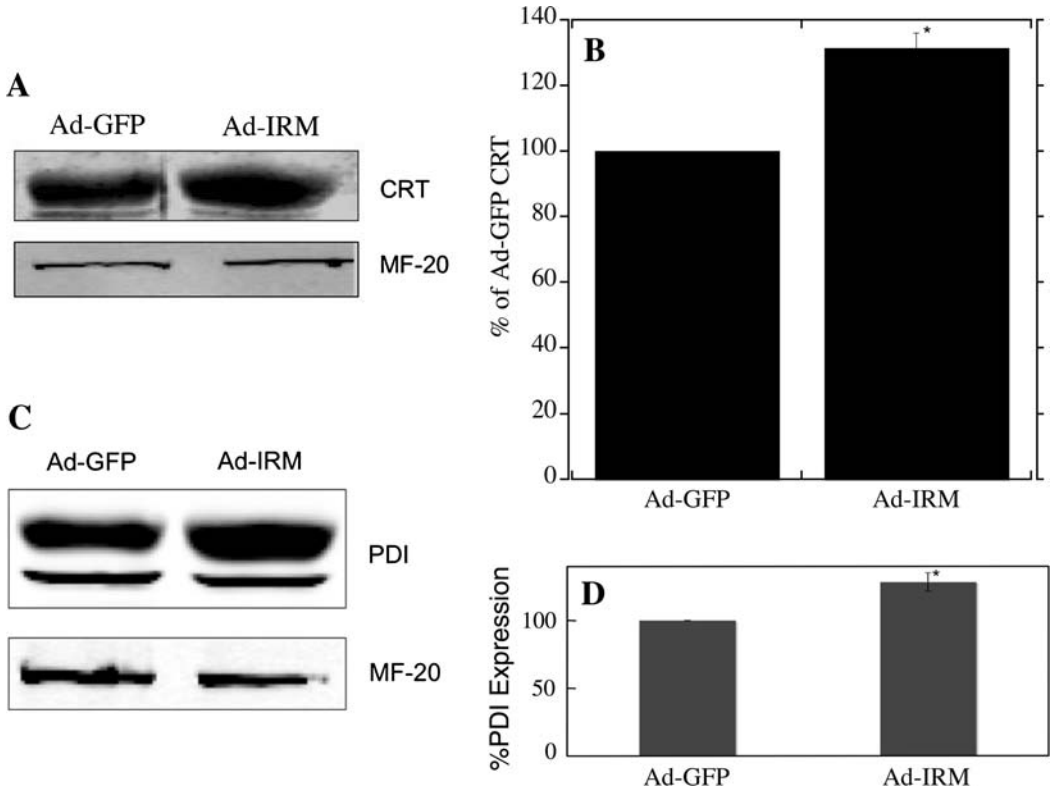

Fig. 6 Western blot analysis of ER stress protein expression levels in cells treated with control adenovirus or adenovirus expressing NHE1 protein. **a** Example of Western blot of cell extracts treated with adenoviral vectors expressing either NHE1 (Ad-IRM) or GFP (Ad-GFP) where indicated. Upper panel Western plot for CRT expression, Lower panel Western blot for myosin heavy chain expression with antibody MF-20. **b** Summary of CRT expression results after correction for Western blotting using myosin heavy chain levels. * Significantly different from control infected cells at $P < 0.05$. **c** Example of Western blot of cell extracts treated with adenoviral vectors expressing either NHE1 (Ad-IRM) or GFP (Ad-GFP) where indicated. Upper panel Western plot for PDI expression, Lower panel Western blot for myosin heavy chain expression with antibody MF-20. **d** Summary of PDI expression results after correction for Western blotting using myosin heavy chain levels. * Significantly different from control-infected cells at $P < 0.05$

further examine the mechanism by which this occurs and methods to prevent these detrimental effects in the myocardium.

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