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Na^+/H^+ exchanger isoform 1 facilitates cardiomyocyte embryonic stem cell differentiation

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Li X, Karki P, Lei L, Wang H, Fliegel L. Na^+/H^+ exchanger isoform 1 facilitates cardiomyocyte embryonic stem cell differentiation. *Am J Physiol Heart Circ Physiol* 296: H159–H170, 2009. First published November 14, 2008; doi:10.1152/ajpheart.00375.2008.—Embryonic stem cells provide one potential source of cardiomyocytes for cardiac transplantation; however, after differentiation of stem cells in vitro, cardiomyocytes usually account for only a minority of cells present. To gain insights into improving cardiomyocyte development from stem cells, we examined the role of the Na^+/H^+ exchanger isoform 1 (NHE1) in cardiomyocyte differentiation. NHE1 protein and message levels were induced by treatment of CGR8 cells to form embryoid bodies and cardiomyocytes. The NHE1 protein was present on the cell surface and NHE1 inhibitor-sensitive activity was detected. Inhibition of NHE1 activity during differentiation of CGR8 cells prevented cardiomyocyte differentiation as indicated by decreased message for transcription factors Nkx2-5 and Tbx5 and decreased levels of α-myosin heavy chain protein. Increased expression of NHE1 from an adenoviral vector facilitated cardiomyocyte differentiation. Similar results were found with cardiomyocyte differentiation of P19 embryonal carcinoma cells. CGR8 cells were treated to induce differentiation, but when differentiation was inhibited by dispersing the EBs, myocardial development was inhibited. The results demonstrate that NHE1 activity is important in facilitating stem cell differentiation to cardiomyocyte lineage. Elevated NHE1 expression appears to be triggered as part of the process that facilitates cardiomyocyte development.

intracellular pH; adenovirus

THE Na^+/H^+ EXCHANGER (NHE) is a ubiquitous plasma membrane glycoprotein that plays a key role in intracellular pH (pH_i) regulation. It extrudes one intracellular proton in exchange for one extracellular sodium, removing excess intracellular acid that is a product of metabolism (14). Nine isoforms have been described, and the NHE isoform 1 (NHE1) is the only plasma membrane isoform present in cardiac tissue (23). NHE1 not only regulates pH_i in the myocardium, it also has been implicated in myocardial ischemia/reperfusion injury (23) and in cardiac hypertrophy following myocardial infarction (3, 21, 25). A large number of studies have shown that inhibition of the NHE during ischemia and reperfusion prevents myocardial injury (4, 11, 24, 32, 45). We and others (9, 13, 22) have shown that NHE inhibition can prevent the deleterious remodeling of the myocardium induced by hypertrophic stimuli.

A number of studies have suggested that the levels of NHE1 vary during growth and differentiation and that increased NHE1 expression may be important in growth and differentiation of cardiomyocytes. That NHE1 is involved in cell growth, proliferation, and cell differentiation was demonstrated in a number of cell types (2, 15, 18, 35, 44), including neuronal differentiation of P19 cells (41). In that case, this involved a 10-fold increase in NHE1 transcription (12). We have also found that NHE is developmentally regulated in the myocardium during development. Transcription from the NHE1 promoter was greatly elevated in the mouse heart embryo during development, and levels of expression of the NHE1 protein were elevated during heart development and immediately after birth (36, 37). NHE1 message and activity have also been shown to be elevated in several models of cardiac hypertrophy (8, 20, 22), which is interesting since cardiac hypertrophy is characterized by a reactivation of the fetal gene program (1). NHE1 protein and activity have also been shown to decline postnatally in the myocardium, when cell differentiation also declines (17, 36). These changes all seem to follow a pattern of increased NHE1 expression in development and differentiation and suggest that NHE1 may be important in these processes in the myocardium.

Recently, studies have suggested that myocyte transplantation may improve cardiac function in different models. One source of cardiac tissue for transplantation is embryonic stem (ES) cells. The ability of ES cells to differentiate into specific cell types holds great potential for the therapeutic use in cell and gene therapy. However, differentiated cardiomyocytes typically account for only a minority of cells present within embryoid bodies (EBs) of ES cells (7, 26, 43). Therefore, it is of interest to determine factors that influence the yield of cardiomyocytes during ES cell differentiation. Because of the role that the NHE1 isoform of the NHE plays in cardiac development and differentiation, we examined its influence on this process. We examined effects of inhibition of NHE1 activity on the differentiation process including effects on expression of different transcription factors important in early cardiac differentiation. Our results are the first report that the NHE is important in the formation of cardiomyocytes in EBs.

MATERIALS AND METHODS

The mouse stem cell line (CGR8) was kindly provided by Dr. Marek Michalak, Department of Biochemistry of University of Alberta. DMEM and ES cell-qualified fetal bovine serum were from Wisent, Canada. Anti-mouse NHE antibody was from BD Biosciences. The monoclonal antibody (MF20) for α-myosin heavy chain (α-MHC) was from the Developmental Studies Hybridoma Bank of the University of Iowa (Iowa City, IA). Angiotensin II was from Sigma (St. Louis, MO). TRIzol reagent, Moloney murine leukemia virus (MMLV), reverse transcriptase, SYBR Green, and *Taq* DNA

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polymerase was from Invitrogen Life Technologies (Carlsbad, CA). Dithiothreitol (DTT) and 3,4,5-dimethyl thiazol-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO) and *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroaniline (Ac-DEVD-pNA) was from Alexis Biochemicals (Lausen, Switzerland). Horseradish peroxidase-conjugated goat anti-mouse IgG was from Bio/Can (Mississauga, ON, Canada). EMD87580 was a generous gift of Merck, Darmstadt, Germany.

ES cell culture propagation and differentiation. CGR8 ES cells, were cultured in gelatin-coated petri-dishes without feeder cells in DMEM supplemented with nonessential amino acids, L-glutamine, β-mercaptoethanol, 10% ES cell-qualified fetal bovine serum, and leukemia inhibitory factor (1:1,000 dilution of recombinant leukemia inhibitory factor) in a humidified 5% CO_2 atmosphere at 37°C. They were maintained at <70% confluency to keep an undifferentiated phenotype (27, 31). Cells were passaged every 2 days. Differentiation of CGR8 cells was performed by the hanging-drop method (29). In brief, adherent cells were enzymatically dissociated by using 0.25% trypsin-EDTA at *day 0* of induction. EBs were formed in hanging drops of 400 cells in 20 μl of differentiation medium with 20% ES cell-qualified fetal bovine serum in the absence of leukemia inhibitory factor. After 2 days, EBs were collected and cultured in suspension for 3 days. After 5 days, EBs were plated on gelatin-coated dishes and the differentiation medium was exchanged every other day. Spontaneously beating cardiomyocytes within EBs were observed beginning on *day 8*. For some experiments, the differentiation medium contained 10 μM EMD87580 or 100 nM angiotensin II beginning from *differentiation day 0* till the end of experiments. In other experiments EBs were treated with 100 nM H_2O_2 in differentiation medium for 2 h at *day 4*. In a series of experiments cells were treated with an adenovirus that expressed a hemagglutinin (HA)-tagged NHE1 protein. The adenovirus containing NHE1 has been described earlier (10). For these experiments cells were infected at a multiplicity of infection of 20 beginning on *day 0*. A control adenovirus was used for these experiments that did not express the NHE1 protein.

P19 mouse embryonal carcinoma cells were obtained from American Type Culture Collection (Bethesda, MD) and were maintained in α-minimum essential medium supplemented with 25 mM $NaHCO_3$, 2.5% fetal bovine serum, and 7.5% newborn calf serum as reported earlier (41). To induce cardiomyocyte differentiation, P19 cells were grown in suspension culture for 4 days in the presence of 1% DMSO and then were plated on tissue culture dishes for a further 2–4 days wherever indicated. In some cases 10 μM EMD87580 was present throughout the differentiation process.

Cytotoxicity assays. Mitochondrial dehydrogenase activity and caspase-3-like activity assays were performed to detect general and apoptotic cell death, respectively. The viability of cells grown in the presence or absence of 10 μM EMD87580 was determined by MTT assay as described previously (33). The method is based on the conversion of a tetrazolium salt MTT to insoluble formazan by mitochondrial dehydrogenases of living cells. Briefly, 20 μl of MTT (5 mg/ml) was added to cells in culture with the fresh medium and incubated for 4 h. The formed formazan crystals were dissolved in 100 μl of lysis buffer (20% sodium dodecyl sulfate in 50% *N*,*N*-dimethyl formamide) and another 12-h incubation was allowed to dissolve the formazan. The absorbance was measured at 570 nm by use of a microplate spectrophotometer (Molecular Devices).

To assay caspase-3-like activity after *day 14*, cells cultured in 60 mm dishes were harvested and washed once with ice-cold phosphate-buffered saline. Then, 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 phenylmethylsulfonyl chloride, 10 μg/ml leupeptin) was added and incubated on ice for 10 min. 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).

SDS-PAGE and immunoblotting. To test for NHE1 and α-MHC expression, immunoblot analysis was used on samples from total cell lysates of CGR8 EBs. Cell lysates were made as described earlier (38). For Western blot analysis, equal amounts of each sample (75 μg total protein) were resolved on 10% SDS-polyacrylamide gels. The gel was transferred onto a nitrocellulose membrane and immunostained with anti-NHE1 or MF20 monoclonal antibody and peroxidase-conjugated goat anti-mouse antibody. The Amersham enhanced chemiluminescence Western blotting and detection system was used to visualize immunoreactive proteins. Densitometric analysis of X-ray films was carried out via NIH Image 1.63 software (National Institutes of Health, Bethesda, MD).

Reverse transcription PCR. Total RNA was extracted from ES cells with TRIZOL reagent, and 1 μg of RNA was used to synthesize one strand of cDNA with MMLV reverse transcriptase. PCR was then performed. Sequences of PCR primers were as follows (5′ to 3′): ccctcacgtgcgcacaccc and gacgtctgattgcaggaagg (mNHE1); gctacaagtgcaagcgacag and gggtaggcgttgtagccata (GATA4); TGCAGAAGGCAGTGGAGCTGGACAAAGCC and TTGCACTTGTAGCGACGGTTCTGGAACCAG (Nkx2-5); CCACTGGATGCGACAACTTGTCTCC and GACGTGGGTGCAAAACGCAGTGTTC (Tbx5); AGATACCCACAACACACCACGCGCC and ATCCTTCAGAGAGTCGCATGCGCTT (MEF2C); GGAACATAGCCGTAAACTGC and TCACTGTGCCTGAACTTACC (β-tubulin), ATTCAACGGCACAGTCAAGG and CAGTGTAGCCCAAGATGCCCT (glyceraldehyde phosphate dehydrogenase). For reverse transcription-PCR, the reaction conditions were as follows: an initial denaturation was at 94°C for 3 min and then 30 to 35 cycles consisting of 94°C for 40 s, 60°C for 40 s, 72°C for 1 min, followed by final extension at 72°C for 7 min.

Real-time PCR. Total RNA was extracted from ES cells, and 1 μg of RNA was used to synthesize one strand of cDNA with MMLV reverse transcriptase. cDNA (1 μl) from reverse transcripts experiments was used for quantitative PCR with the SYBR Green as double-stranded DNA binding dye with Rotor-Gene equipment (RG-3000, Corbett Research, Montreal Biotech). Sequences of PCR primers for MEF2C, Tbx5, NKx2-5, GATA4, mNHE1, and β-tubulin were same as RT-PCR. The transcript for β-tubulin or glyceraldehyde phosphate dehydrogenase was used for internal normalization.

Cell surface expression. Cell surface expression was measured essentially as described earlier (38, 39). Briefly, cells were labeled with Sulpho-NHS-SS-Biotin (Pierce Chemical, Rockford, IL) and immobilized streptavidin resin was used to remove cell surface-labeled NHE. Equivalent amounts of the total and unbound proteins were analyzed by SDS-PAGE, and Western blotting was as described above. The relative amount of NHE1 on the cell surface was calculated by comparing both the 110-kDa and the 95-kDa species of NHE1 in Western blots of the total and unbound fractions. We have found earlier that biotin-labeled protein does not reliably dissociate from the streptavidin beads, even in the presence of SDS (39), and therefore did not include the streptavidin-bound fraction.

Na^+/H^+ exchange activity. Na^+/H^+ exchange activity of EBs was measured with a PTI Deltascan spectrofluorometer connected with a D104 Microscope Photometer and a Leica DMIRB microscope with a ×10 objective. Intracellular pH (pH_i) was measured by use of 2′,7-bis (2-carboxyethyl)-5 (6) carboxyfluorescein-AM (BCECF-AM; Molecular Probes, Eugene, OR) (19). The initial rate of Na^+-induced recovery of pH_i was measured after acute acid load induced by ammonium chloride. Firstly, the EB was plated on a gelatin-coated round coverslip at *day 5* after differentiation began. At *day 8* or *day 12*, the EB coverslip was loaded with BCECF-AM (3 μg) in 400 μl serum-free DMEM medium for 20 min in a temperature controlled perfusion chamber (Warner, Hamden, CT) at 37°C in the dark. The BCECF-AM was removed using a continuous flow of normal buffer

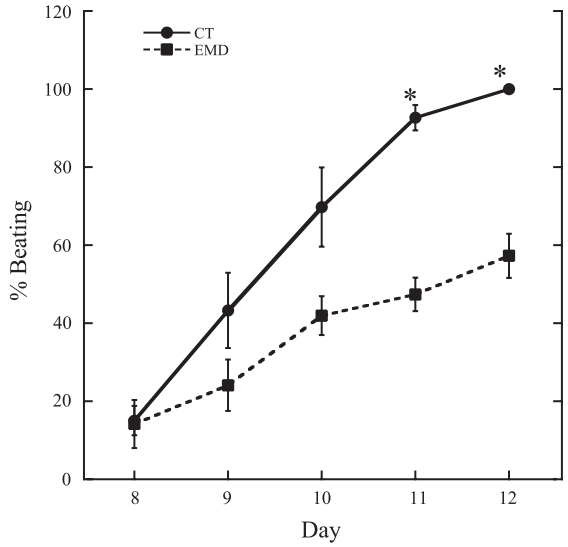


Fig. 1. Effect of EMD87580 treatment on percentage of beating embryoid bodies (EBs) during CGR8 differentiation. EBs were prepared as described in MATERIALS AND METHODS in the presence (EMD) and absence (Control; CT) of 10 μM EMD87580. The percentage of beating EBs was then quantified by visual observations from *day 8* onward. Results are means ± SE of 4 experiments. *Significantly different from the untreated cells at $P < 0.05$.

(135 mM NaCl, 4.5 mM KCl, 2 mM $CaCl_2$, 20 mM HEPES, and 10 mM glucose, pH 7.4). Ammonium chloride (50 mM × 3 min) was used to transiently induce an acid load, and the recovery in the presence of 135 mM NaCl was measured as described previously after ammonium chloride removal (19). For measurement of internal pH of EBs cells were maintained in a flow chamber with a volume of less than 1 ml with a flow rate of 3 ml per minute. To test the effect of EMD87580 on activity of the NHE1, we used the standard NHE assay, and the initial rate of Na^+-induced recovery of pH_i was measured in the presence of 10 μM EMD87850.

For some experiments, the EB was dissociated with trypsin-EDTA at *day 5*, and the dispersed cells were plated and maintained on rectangular coverslips. At *day 8* or *day 12*, ammonium chloride (50 mM × 3 min) was used to transiently induce an acid load and the recovery was measured in the presence of 135 mM NaCl as described above. Assay of NHE1 activity was as described earlier (39). The effect of EMD87580 on activity of the dispersed cells was measured as described above.

Results are shown as means ± SE, and statistical significance was determined by a Mann-Whitney *U*-test or a Student's *t*-test (Fig. 6*C*).

RESULTS

Initial experiments examined the effect of treatment with the NHE1-specific inhibitor EMD87580 (22) on the percentage of beating EBs present. This was used as a marker of cardiomyocyte differentiation as described earlier (7, 26, 43). The

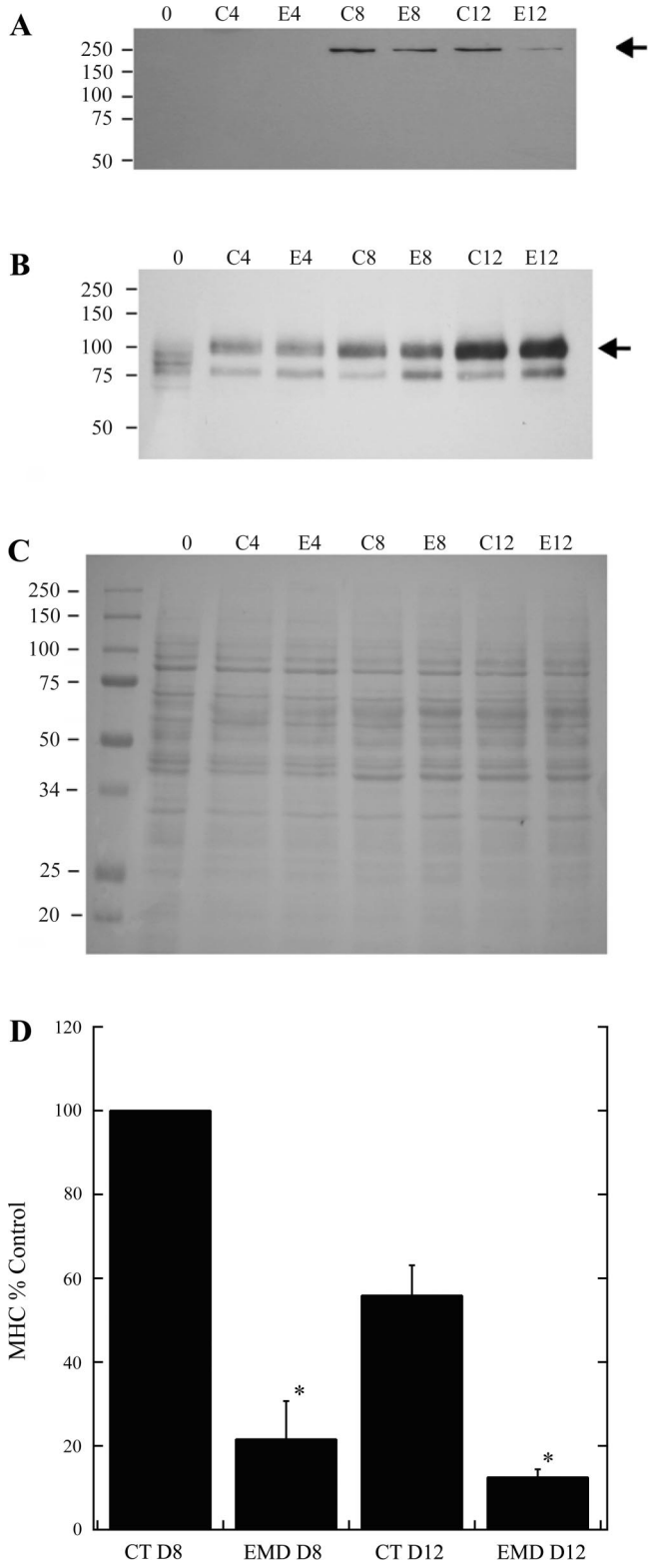
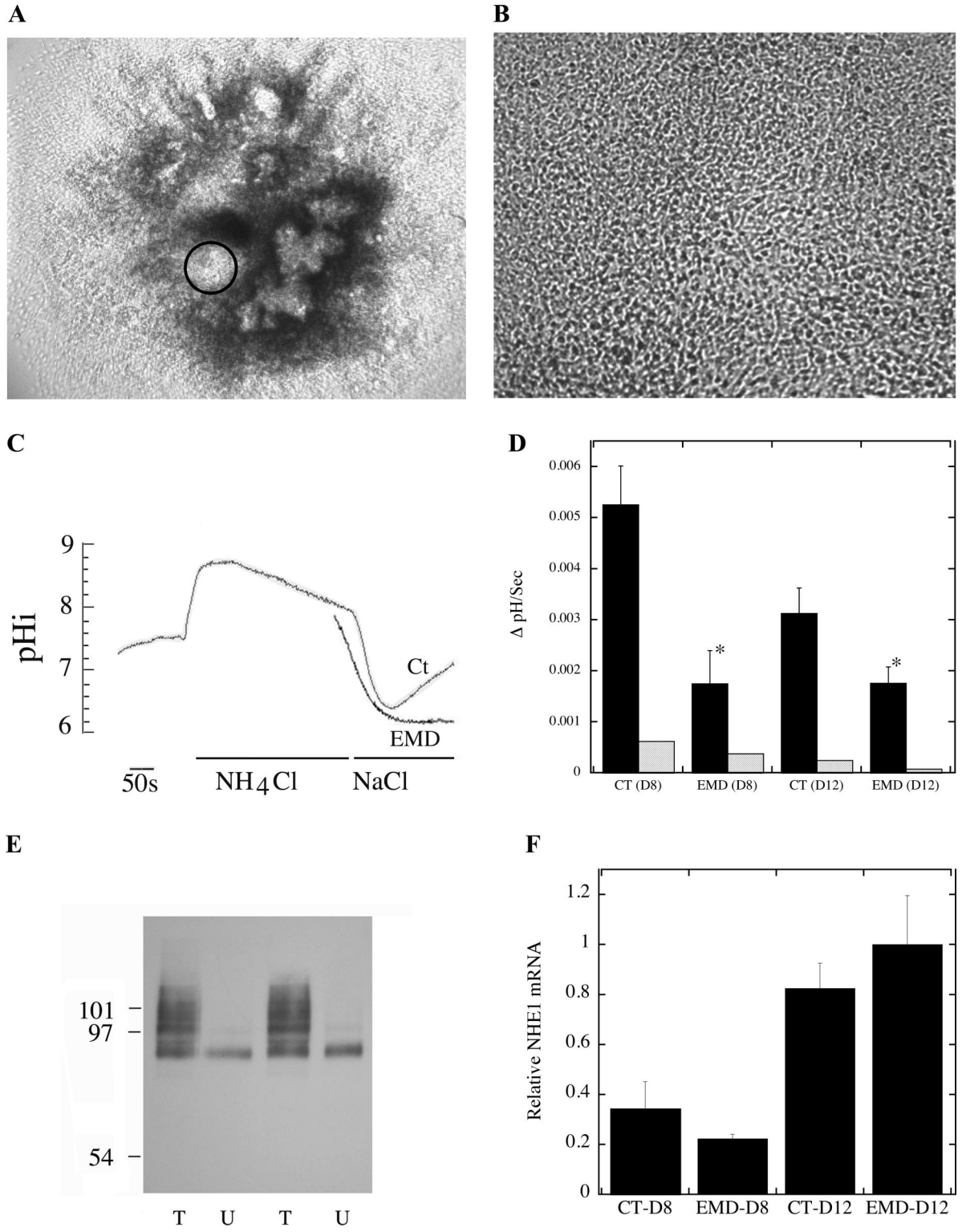


Fig. 2. Effect of EMD87580 treatment on α-myosin heavy chain (α-MHC) and Na^+/H^+ exchanger isoform 1 (NHE1) expression during CGR8 differentiation to EBs. CGR8 cells were treated so as to induce differentiation to EBs in the presence and absence of 10 μM EMD87580. *A*: Western blot analysis of cell extracts examined for the presence of α-MHC protein. Arrow denotes location of immunoreactive protein. 0, *day 0*; C4, *day 4* control EBs; E4, EMD87580-treated 4-day-old EBs; C8, E8, C12, and E12 as for C4 and E4 but on *days 8* and *12*, respectively. *B*: Western blot analysis of cell extracts examined for the presence of NHE1 protein. Arrow denotes location of fully glycosylated NHE1 protein. *C*: Ponceau S stain of electrophoretic transfer of proteins examined for NHE1 and α-MHC expression. Results are typical of at least 5 experiments. *D*: summary of effect of EMD87580 on expression of α-MHC protein. α-MHC protein levels were measured from Western blots as in *A* and corrected for protein levels transferred to nitrocellulose membranes. Results are means ± SE of at least 3 determinations; *significantly different from untreated cells at $P < 0.05$.

results are shown in Fig. 1. Treatment with 10 μM EMD87580 reduced the percentage of beating EBs at all times from *day 8* to *12*. By *day 12* only approximately half of the treated EBs were beating compared with the control.

We examined the effect of NHE inhibition on NHE1 expression and on expression of α-MHC during EB differentiation. Figure 2 illustrates a typical Western blot. Treatment with EMD87580 resulted in a greatly decreased expression of α-MHC protein especially at *day 12* (Fig. 2*A*) whereas the level of NHE protein remained constant at different days of treatment among the control and EMD87580-treated cells (Fig. 2*B*). Equal amounts of protein were added for SDS-PAGE and the blots were routinely stained with Ponceau S to confirm that equal amounts of protein were loaded and transferred (Fig.



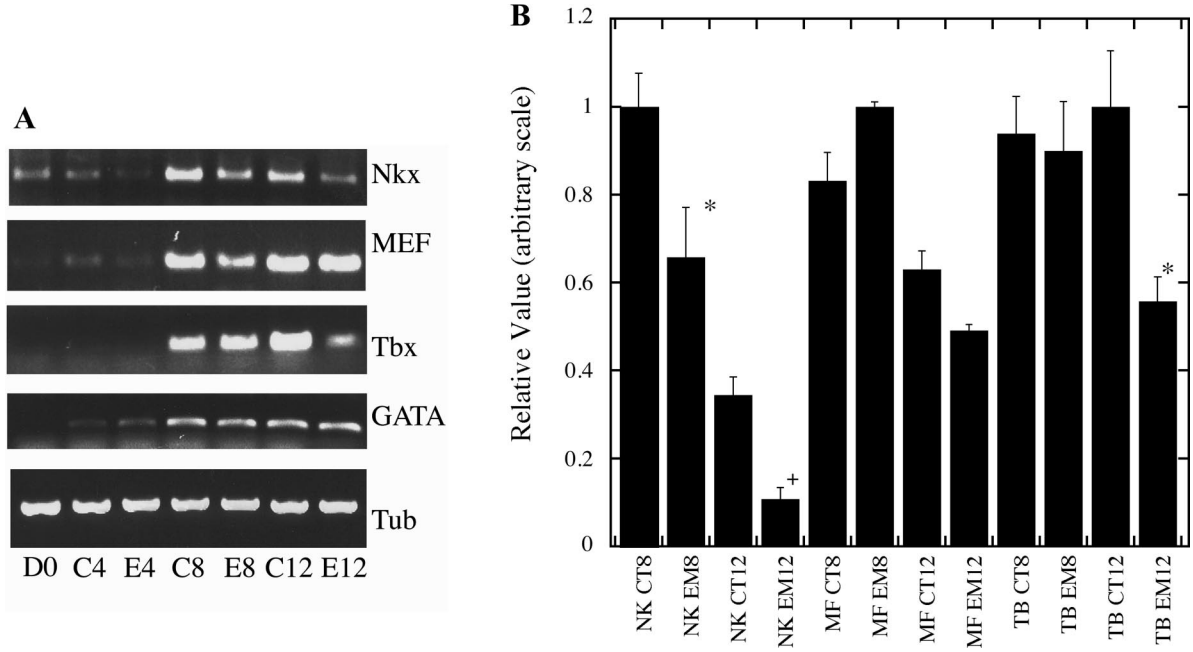


Fig. 4. RNA levels of cardiomyocyte marker transcription factors in EBs after treatment with EMD87580. *A*: photograph of PCR products using RT-PCR as described in MATERIALS AND METHODS. D0, *day 0* EBs. Nkx, Nkx2-5; MEF, MEF2C; Tbx, Tbx5; GA, GATA4, Tub, β-tubulin. *B*: measurement of mRNA levels using real-time PCR. EM, EMD87580-treated EBs; 8 and 12, 8- and 12-day-old EBs, respectively. NK, NKx2-5; MF, MEF2C; TB, Tbx5. $*P < 0.05$ and $+P < 0.01$, chronically EMD87580-treated value is significantly different from the corresponding value of untreated cells.

2*C*). Figure 2*D* summarizes the effects of EMD87580 on α-MHC protein levels. At either *day 8* or *day 12* EMD87580 caused significant decreases in the level of α-MHC protein.

We determine the activity of the NHE using the fluorescent indicator BCECF. Beating areas of the EB were chosen using a microscope and the pH_i was determined as described in MATERIALS AND METHODS. Figure 3*A* shows a region typical of the EB that was beating and in which we determined pH_i. Figure 3*B* shows an enlargement of such an area. We found that pH_i could be accurately monitored by via this microscope-based system. Figure 3*C* demonstrates that ammonium chloride induced an initial alkalinization from neutral pH, followed by a slow acidification. When ammonium chloride was removed, there was a rapid acidification followed by a recovery in NaCl-containing medium. In the presence of the specific NHE1 inhibitor EMD87580, the recovery from acid load was virtually completely blocked. We examined the activity of the NHE in differentiating EBs that were chronically treated with the NHE inhibitor EMD87580. The activity was then determined in the presence or absence of 10 μM EMD87580. Inhibitor sensitive NHE activity was present in all EBs tested. The highest level of activity was present in 8-day-old EBs. Activity declined in 12-day-old EBs. Chronic treatment with EMD87580 resulted in decreased NHE activity even when the inhibitor was not added to the assays. In all groups tested (Fig. 3*D*), 10 μM EMD87580 inhibited virtually all the NHE activity.

We also examined the surface targeting of the NHE in 8-day-old EBs. Cells were labeled with Sulpho-NHS-SS-Biotin, and immobilized streptavidin resin was used to remove cell surface labeled NHE. Western blotting was then used to examine the NHE1 protein present in the total fraction and in the fraction unbound to the streptavidin resin. The results (Fig. 3*E*) demonstrate that a large majority of the protein is present on the extracellular surface. In the total fraction, the NHE1 protein was present in large majority as fully glycosylated protein greater than 100 kDa in size. There was also a smaller immunoreactive species that likely represents incompletely glycosylated or unglycosylated NHE1 protein. The fraction which was not bound to streptavidin resin contained virtually no larger, glycosylated form of the protein and only partially or deglycosylated NHE1 protein, which could be originating from intracellular NHE1 in the process of biosynthesis (38).

Real-time PCR was used to determine mRNA levels for NHE1 in EBs either treated or untreated with EMD87580. Treatment with EMD87580 did not result in any change in the

Fig. 3. Characterization of the Na^+/H^+ exchanger in EBs. *A*: photograph of EBs at *day 8* (magnification ×25) indicating typical beating area used for intracellular pH (pH_i) determination. *B*: enlargement of area used for pH_i determination (×100). *C*: characterization of pH_i in EBs subjected to ammonium chloride-induced acidosis in the presence or absence of 10 μM EMD87580. Short bar indicates 50 s. NH_4Cl indicates addition of 50 mM ammonium chloride. NaCl indicates removal of ammonium chloride-containing medium and return of "normal buffer" containing 135 mM NaCl. The trace of pH_i in the presence of EMD is offset for clarity, and only the acidification induced by ammonium chloride and recovery are illustrated. *D*: Na^+/H^+ exchanger activity of 8- (D8) or 12-day-old (D12) EBs either untreated or chronically treated with 10 μM EMD87580. Assays were done as described for *C* in the presence (shaded bars) or absence (solid bars) of 10 μM EMD87580. *Chronically EMD87580-treated value is significantly different from the corresponding value of untreated cells. *E*: representative Western blot for measurement of the subcellular localization of the Na^+/H^+ exchanger in 8-day-old EB. Cells were treated with Sulpho-NHS-SS-Biotin and solubilized, and biotin-labeled proteins were bound to streptavidin agarose beads as described in MATERIALS AND METHODS. A sample of the total cell lysate (T) and an equivalent amount of unbound lysate (U, intracellular) were run on SDS-PAGE. Western blotting with an anti-NHE1 antibody was used to identify NHE1 protein. *F*: real-time PCR measurement of NHE1 mRNA levels of 8- or 12-day-old EBs either untreated or treated with EMD87580.

levels of mRNA relative to untreated EBs. There was an apparent increase in NHE1 message levels from *day 8* to *day 12*, but this was unaffected by EMD87580 treatment (Fig. 3*F*).

Because Western blot analysis and analysis of the rate of EB beating suggested that NHE inhibition reduced the rate of EB differentiation to cardiomyocytes, we examined the expression levels of several transcription factors that are important to cardiomyocytes in cells treated with NHE inhibitor. Initial experiments used RT-PCR. We found that the levels of Nkx2.5 were reduced with EMD87580 treatment in both 8- and 12-day-old EBs. There was some reduction in MEF2C levels in 8-day-old treated EBs and in Tbx5 levels in 12-day-old treated EBs. GATA 4 levels and β-tubulin levels were unaffected (Fig. 4*A*). We also performed real-time PCR to more accurately examine the message levels. The results are summarized in Fig. 4*B*. PCR was successful for Nkx2.5, MEF2C, mNHE1, and Tbx5. Analysis of GATA4 was not successful for unknown reasons. Treatment of EBs with EMD87580 resulted in a decline in the levels of several of the markers of cardiomyocyte differentiation. At *day 12*, the level of all three markers was decreased, most notably that of Nkx2.5 and Tbx5. There was also a slight decrease in the level of Tbx5 and Nkx2.5 at *day 8*. There was no significant difference in the level of mNHE1 with EMD87580 treatment in both 8- and 12-day-old EBs (not shown). Prior to *day 8* there were not high enough levels of these transcription factors for accurate quantification.

We examined the NHE activity and differentiation of dispersed cells of EBs dissociated at *day 5* and then plated and maintained on rectangular coverslips. At *day 8* or *day 12*, NHE activity, NHE1 and α-MHC expression were determined. Figure 5*A* illustrates examples of the rate of recovery from an ammonium chloride-induced acidosis of 8-day-old dispersed CGR8 cells. The cells were acidified by ammonium chloride

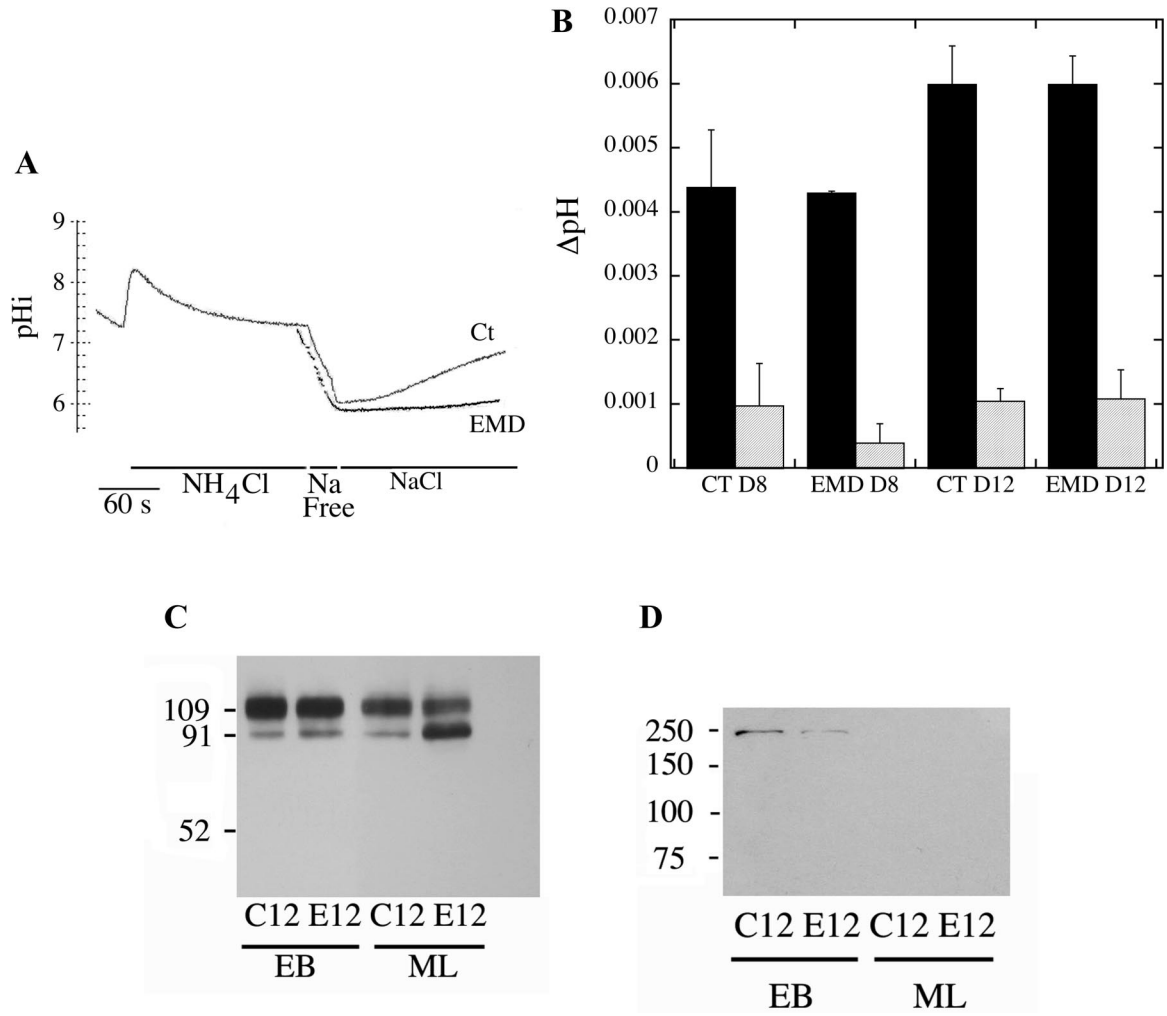


Fig. 5. Characterization of Na^+/H^+ exchanger activity of dispersed CGR8 cells. *A*: examples of pH_i recovery from acid load in monolayers of 8-day-old CGR8 cells subjected to ammonium chloride-induced acidosis in the presence (EMD) or absence (Ct, control) of 10 μM EMD87580. Bar indicates 60 s. NH_4Cl indicates addition of 50 mM ammonium chloride. Na Free indicates a brief period of incubation in Na^+-free buffer. NaCl indicates removal of ammonium chloride-containing medium and return of "normal buffer" containing 135 mM NaCl. The trace of pH_i in the presence of EMD is slightly offset for clarity. *B*: summary of Na^+/H^+ exchanger activity of dispersed CGR8 cells either untreated (CT) or chronically treated (EMD) with 10 μM EMD87580. Assays were done as described for Fig. 3*C* in the absence (solid bars) or presence (shaded bars) of 10 μM EMD87580. *C*: Western blot analysis of 12-day-old cell extracts examined for the presence of NHE1 protein. Samples were either untreated (C12) or chronically treated (E12) with 10 μM EMD87580. EB, cell extracts from embryoid bodies; ML, cell extracts from dispersed monolayers of CGR8 cells. *D*: Western blot analysis of 12-day-old cell extracts examined for the presence of α-MHC protein. Samples were as described in *C*.

and pH_i recovered in the presence of NaCl. EMD87580 blocked the recovery in NaCl containing medium. Figure 5*B* summarizes the results of a series of experiments measuring activity of the NHE in dispersed cells either untreated or chronically treated with EMD87580. NHE activity was present in all samples and increased slightly in 12-day-old cells. Chronic treatment with EMD87580 did not affect NHE1 activity whereas acute use during the assay inhibited the activity to a very low level. Western blot analysis examined expression of the NHE1 protein. Figure 5*C* demonstrates that NHE1 expression was maintained, although slightly declined in the dispersed CGR8 cells, compared with the intact EBs. The amount of unglycosylated or partially unglycosylated NHE1 protein increased in the 12-day-old dispersed cells. Expression of the α-MHC protein was examined in samples from intact EBs and dispersed CGR8 cells (Fig. 5*D*). The expression of α-MHC protein was not apparent in the dispersed cells. Chronic EMD87580 reduced the level of α-MHC protein in EBs of CGR8 cells.

We also examined the effect of treatment of EBs with angiotensin II and H_2O_2, which are known to stimulate the cardiomyocyte differentiation process (28). The combination of H_2O_2 and angiotensin II together increased the proportion of beating EBs. Treatment with either alone had either no or

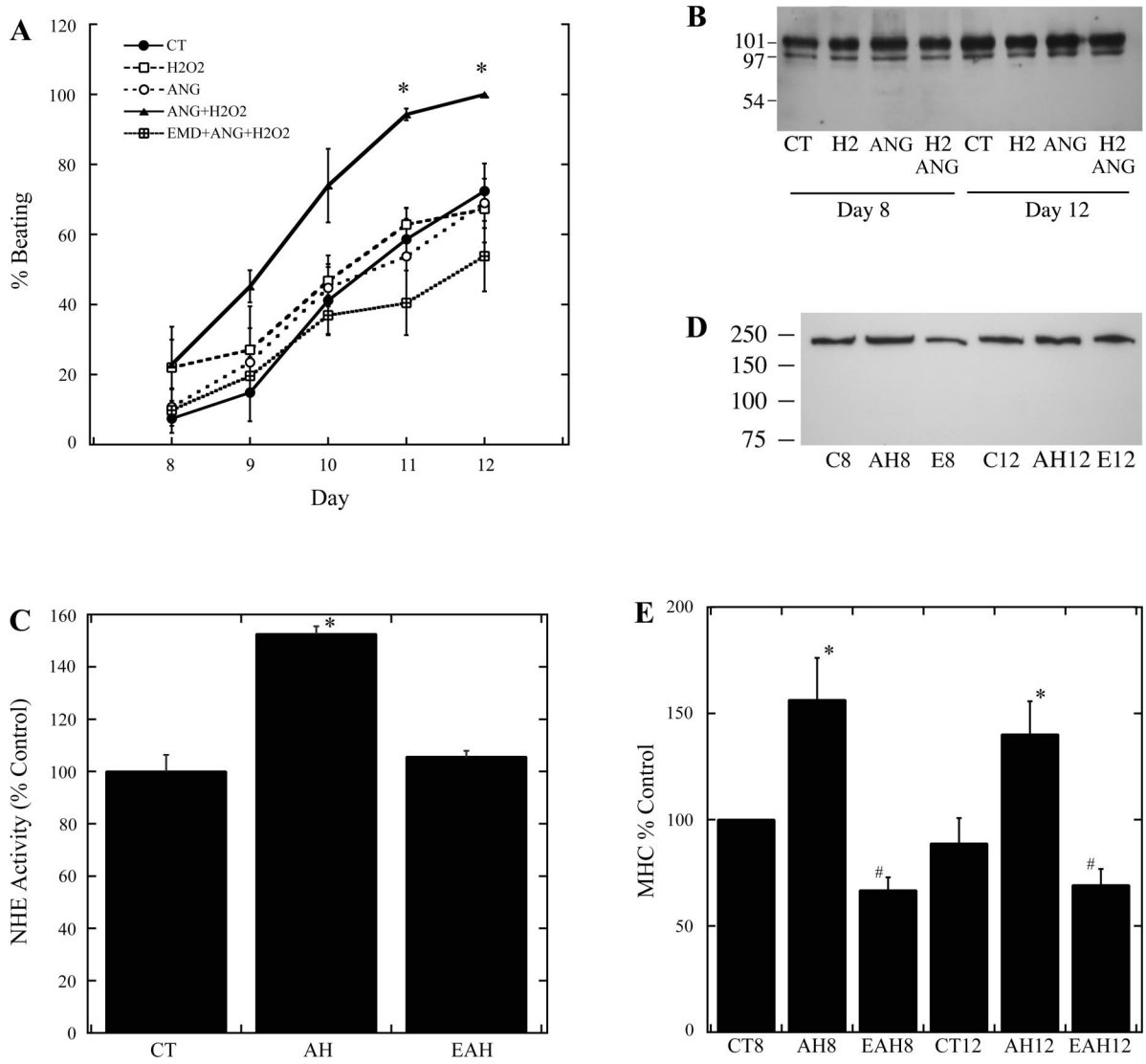


Fig. 6. Effect of angiotensin II and H_2O_2 treatments on EB cardiomyocyte differentiation. EBs were prepared as described in MATERIALS AND METHODS and treated with H_2O_2 (100 nM, 2 h at *day 4*) or angiotensin II (100 nM from *day 0* on). *A*: effect of treatments on percentage of beating EBs. *Significantly different from untreated cells at $P < 0.05$. *B*: Western blot analysis of cell extracts examined for the presence of NHE1 protein after angiotensin II and/or H_2O_2 treatments. H2, H_2O_2 treated; Ang, angiotensin II treated. Samples from *day 8* or *12* are indicated. *C*: NHE1 activity of control and H_2O_2- and angiotensin II-treated (AH) EBs. EAH, H_2O_2 and angiotensin II treated in the presence of EMD87580 10 μM. Activities were measured in 8-day-old EBs as indicated. *Significantly different from untreated cells at $P < 0.05$. *D*: Western blot analysis of cell extracts examined for the presence of α-MHC protein after angiotensin II and H_2O_2 ± EMD87580 treatments. EBs were harvested at *day 8* or *12* as indicated. C, control; AH, treated with angiotensin II and H_2O_2; E, treated with angiotensin II and H_2O_2 in the presence of 10 μM EMD87580. *E*: summary of effect of angiotensin II and H_2O_2 on expression of α-MHC protein on *day 8* and *day 12* EBs. EBs were in the presence (E) or absence of 10 μM EMD87580. α-MHC protein levels were measured from Western blots and corrected for protein levels transferred to nitrocellulose membranes. Results are means ± SE of at least 3 determinations. *Significantly different ($P < 0.01$) from the equivalent age of EBs not treated with angiotensin II and H_2O_2. #Significantly different ($P < 0.05$) from the equivalent age of angiotensin II- and H_2O_2-treated EBs in the absence of EMD87580.

minor effects on the proportion of beating EBs (Fig. 6*A*). Western blot analysis was used to examine the effects of angiotensin II and H_2O_2 on NHE1 protein expression. Figure 6*B* illustrates that there was no significant effect on NHE1 protein expression at either *day 8* or *day 12*. We examined NHE1 activity in control and angiotensin II-H_2O_2-treated EBs. At *day 8* we saw approximately a 60% increase in activity (Fig. 6*C*). Chronic treatment with EMD87580 abolished this effect. Treatment with H_2O_2 and angiotensin II together did, however, result in an increase in α-MHC expression indicative of further cardiomyocyte differentiation (Fig. 6, *D* and *E*).

We characterized the status of cell death in EMD87580-treated and untreated 12-day-old EBs to ensure that any changes we observed were not due to effects of any form of cell death. We performed MTT assays to detect the general cell death and caspase-3-like activity, as a marker of apoptotic cell death. Figure 7 shows that both cell viability, as determined by MTT assay, and caspase 3-like activity were not affected by EMD87580 treatment.

To examine whether inhibition of NHE activity affected differentiation to cardiomyocytes in another cellular model, we used P19 embryonal carcinoma cells. When treated with DMSO they differentiate into cardiac cells (30). Figure 8 illustrates the effects of EMD87580 on the morphology of DMSO-treated P19 cells. Figure 8, *A* and *B*, shows that many of the cells grown in suspension in the presence of DMSO and in the absence of EMD87580, displayed a more pyramidal-like structure. P19 cells have been shown to have significant changes in morphology with differentiation to cardiomyocytes (6). In the absence of DMSO, a low rate of spontaneous differentiation was present (not shown). In contrast to the effects in *A* and *B*, Fig. 8, *C* and *D*, shows that when NHE activity was inhibited with EMD87580 the cells did not have this typical shape and instead grew as undifferentiated aggregates.

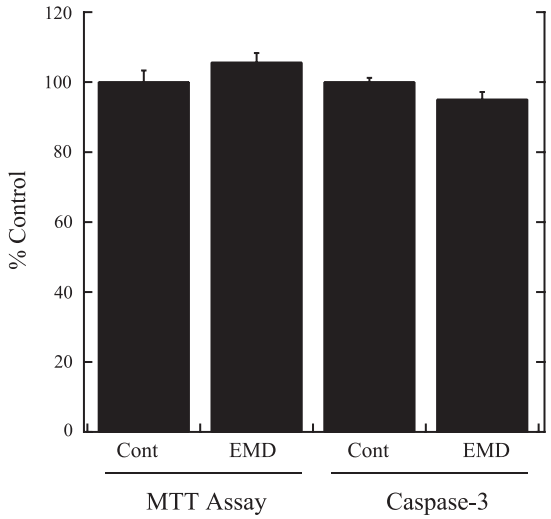


Fig. 7. Effect of EMD87580 on cell survival and caspase-3-like activity of EBs. EBs were prepared as described in MATERIALS AND METHODS and treated with or without (Cont) 10 μM EMD8750 for their entire differentiation period and harvested at *day 12*. Cell survival was measured by the 3,4,5-dimethyl thiazol-2,5-diphenyl tetrazolium bromide (MTT) assay and caspase-3-like activity was assayed as described in MATERIALS AND METHODS. Results are means ± SD of at least 3 determinations; EMD87580-treated cells were compared with untreated cells.

To confirm that EMD87580 treatment affected differentiation to cardiomyocyte-like cells, we performed real-time PCR. The results are shown in Fig. 8*E*. Cells not treated with DMSO showed little NHE1 message and no message for any of the cardiomyocyte markers. In contrast, the message for NHE and all cardiomyocyte markers was increased greatly after 2 and 4 days of DMSO treatment. The levels of the transcription factors Nkx2.5, MEF2C, and GATA4 were all greatly increased by DMSO treatment. When the cells were treated with DMSO in the presence of EMD87580, this effect was greatly reduced, almost to the level of cells not treated with DMSO.

Figure 9 illustrates the effects of exogenous NHE1 expression on a number of parameters of CGR8 cells. Figure 9*A* demonstrates that cells infected with adenovirus containing NHE1 had additional NHE activity compared with cells infected with a control adenovirus. Figure 9*B* demonstrates that adenoviral infection of NHE1 with adenovirus containing an HA-tagged NHE1 caused expression of the protein. The full-length glycosylated protein was evident, as was a deglycosylated or partially glycosylated form that we typically find present (39). Figure 9*C* shows that infection of cells with adenovirus that contains NHE1 increased the percentage of beating EBs. Furthermore, Fig. 9*D* shows that the same treatment elevated the levels of several markers of cardiomyocyte differentiation. Assays of cell viability were done on control infected cells and NHE1 infected cells. There was no difference in viability between these groups (not shown).

DISCUSSION

A number of studies have suggested that the NHE1 is involved in cardiomyocyte growth and differentiation (8, 20, 22, 36, 37). In the present report, we examined whether NHE1 activity has an important role in differentiation of stem cells to cardiomyocytes. We used two different cell lines, CGR8 and P19 cells, to determine the role of NHE1 in this processes. P19 cells, although not strictly an ES cell, are a mouse embryonal carcinoma cell line that can differentiate to all three germ layers and have been used in many developmental studies (5). In both cell types, our results supported the hypothesis that NHE1 activity is necessary for efficient differentiation into cardiomyocyte-like cells. Inhibition of NHE1 activity with the inhibitor EMD87580 resulted in decreased formation of cardiomyocytes as indicated by expression of cardiomyocyte specific mRNA. For CGR8 cells we also documented decreased expression of cardiomyocyte-specific α-MHC protein and decreased contractile behavior with NHE1 inhibition. Conversely, increased expression of NHE1 facilitated cardiomyocyte differentiation.

We documented the presence of the NHE1 isoform of the NHE in CGR8 cells. Western blot analysis with NHE1-specific antibody showed that the protein increased from a barely detectable level to relatively high levels in CGR8 stem cells that had been treated to differentiate toward cardiomyocytes. mRNA levels also increased with treatment to induce differentiation to cardiomyocytes. In addition, the NHE1 activity present in these cells was inhibited by relatively low concentrations of EMD87580, characteristic of this isoform of the NHE and the protein was present predominantly on the cell surface. Although treatment of cells with the EMD87580 inhibited the activity of NHE1 and the differentiation process,

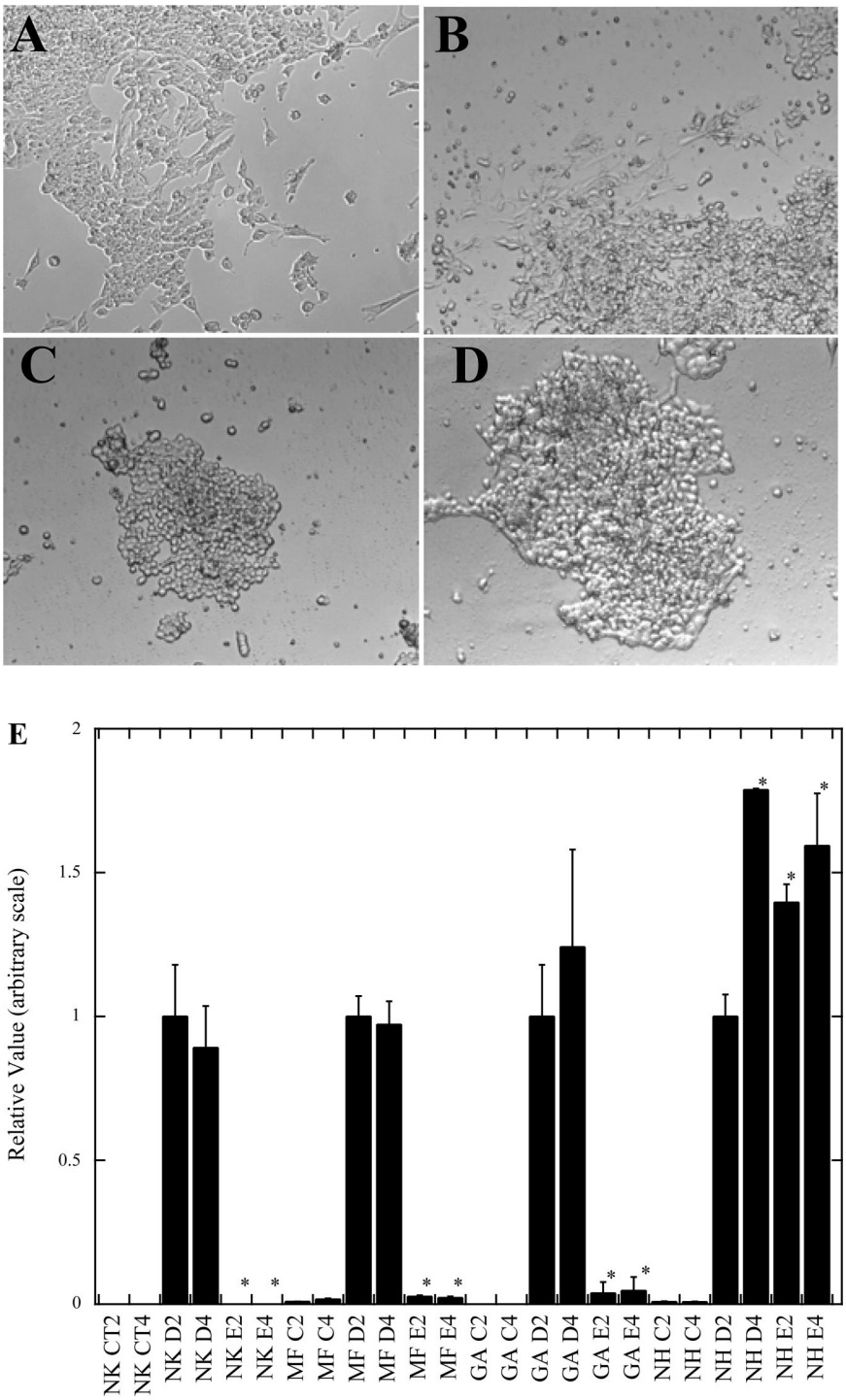


Fig. 8. Effect of EMD87580 on DMSO induced cell differentiation of P19 cells. *A–D*: P19 cells were grown in suspension for 4 days in the presence of 1% DMSO and then transferred to fresh medium for either 2 (*A* and *C*) or 4 days (*B* and *D*) in the absence (*A* and *B*) or presence (*C* and *D*) of 10 μM EMD87580. *E*: effect of NHE1 inhibition on RNA levels of cardiomyocyte markers in EBs. mRNA levels were measured relative to β-tubulin levels by using real-time PCR as described in MATERIALS AND METHODS. P19 cells were grown in suspension for 2 or 4 days in the absence (CT2, CT4) or presence (D2, D4) of 1% DMSO. Where indicated, cells were grown for 2 or 4 days in the presence of 1% DMSO plus 10 μM EMD87580. mRNA levels were measured for Nkx2-5 (NK); MEF2C (MF), and NHE1 (NH). Results are means ± SE of at least 3 experiments. *Significantly different from non-EMD87580-treated cells at $P < 0.05$.

it did not block the changes in NHE1 message levels that occurred. This indicates that the changes in NHE1 expression are independent of the cardiomyocyte differentiation process, triggered earlier, and not caused by the process of differentiation itself. More likely, changes in NHE1 levels are caused by earlier induced changes in gene expression, which are designed to facilitate the differentiation process.

CGR8 cells require cell aggregation and EB formation to allow the complex differentiation process that results in formation of various lineages. In some experiments, the CGR8 cells were dispersed into a monolayer after initial treatment to induce cardiomyocyte differentiation. This was designed to prevent the required structural and functional relationship to other cells and extracellular matrix proteins and to lead to the inhibition of cardiomyocyte differentiation (34). In this case, cardiomyocytes did not form, as indicated by the cell morphology and the protein markers. However, the NHE1 protein was still present as indicated by Western blot analysis and activity. This further indicated that the changes in NHE1 expression are induced prior to differentiation, likely to help

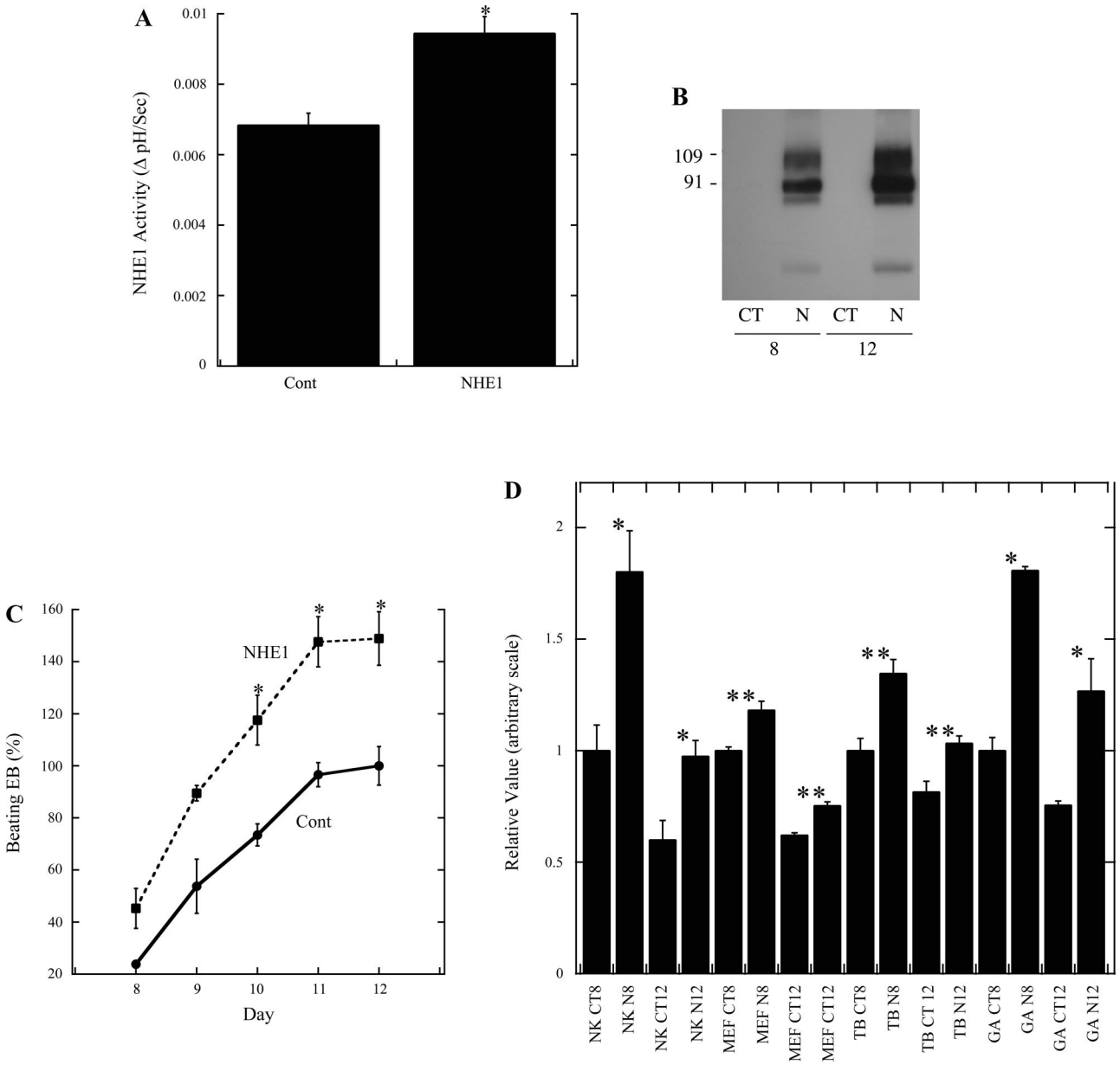


Fig. 9. Effect of adenoviral expression of the Na^+/H^+ exchanger on EB cardiomyocyte differentiation of CGR8 cells. EBs were prepared and treated with control adenovirus or NHE1-expressing adenovirus as described in MATERIALS AND METHODS. *A*: effect of NHE1 expression on Na^+/H^+ exchanger activity of EBs. Na^+/H^+ exchanger activity of *day 8* EBs was measured after 8 days of treatment with control or NHE1-containing adenovirus. *Significantly different from untreated cells at $P < 0.05$. *B*: Western blot against cell extracts of adenovirus-infected EBs. Cell proteins were extracted and immunoblotted for the hemagglutinin (HA) tag present in NHE1 present in the adenovirus. Samples were from 8- or 12-day-old EBs as indicated. CT, control EBs treated with control adenovirus; N, EBs treated with NHE1-expressing adenovirus. *C*: effect of NHE1 expression on percentage of beating EBs. *Significantly different from untreated cells at $P < 0.01$. *D*: effect of NHE1 overexpression on RNA levels of cardiomyocyte marker transcription factors in EBs. mRNA levels were measured by real-time PCR. *Significantly different from untreated cells at $P < 0.05$; **significantly different from untreated cells at $P < 0.01$.

facilitate this process. Inhibition of NHE1 activity during differentiation was inhibitory to cardiomyocyte differentiation. The mechanism by which this occurred is not clear as yet, but it might involve a failure to remove increased acid production of myocardial cells, which could lead to altered further expression of transcription factors.

Although it is apparent that NHE1 plays a facilitative role in cardiomyocyte differentiation, it is also clear that it is not the sole regulator of such differentiation. For example, with inhibition of NHE1, CGR8 cells still showed the presence of cardiomyocyte transcription factors and α-MHC protein and still beat, although all at lower degrees than their untreated counterparts. We also found that NHE1 was present in dispersed CGR8 cells, but without proper structural and functional relationships with other cells, cardiomyocyte differentiation did not occur. NHE1 thus appears to play a facilitative although not essential role in cardiomyocyte differentiation, and other factors are involved.

Treatment of EBs with H_2O_2 and angiotensin improved the differentiation process, as indicated by the percentage of EBs that were beating and expression levels of α-MHC (Fig. 6).

Although the levels of NHE1 protein did not change, the rate of recovery from an acute acid load was increased, suggestive of greater NHE activity. It has previously been shown that both H_2O_2 and angiotensin stimulate NHE1 activity. H_2O_2 has been shown to activate NHE1 through ERK1/2-dependent pathways (42), as has angiotensin (16). It is possible that angiotensin and H_2O_2 stimulation of NHE1 activity through regulatory pathways is responsible for improved differentiation to cardiomyocytes. Similarly, we found that the addition of exogenous NHE1 improved cardiomyocyte development from stem cells. Although it is not clear yet whether this represents a practical way to improve cardiomyocyte differentiation from stem cells, future experiments may explore this possibility.

In P19 cells we previously showed that the NHE1 protein is critical for retinoic acid-induced differentiation to neuronal cells (41). Our present findings that it is also important in differentiation to cardiomyocytes support the idea that the protein is important in differentiation of many cell types. However, NHE1 activity is not universally required for cell differentiation (40). In the myocardium, we showed that NHE1 expression is elevated in the myocardium early in development in utero (36). This supports the observation that it is critical to development in this tissue, which is in agreement with this study. At the present time, it is unknown whether elevated expression or stimulation of NHE1 activity would facilitate a greater percentage of embryonic stem cells to differentiate to cardiomyocytes. Future experiments may explore this possibility.

GRANTS

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