Sodium Hydrogen Exchange 1 (NHE-1) Regulates Connexin 43 Expression in Cardiomyocytes via Reverse Mode Sodium Calcium Exchange and JNK Dependent Pathways

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Abbreviations: cariporide, 4-isopropyl-3-(methylsulfonyl)benzoyl-guanidine methanesulfonate; Cx43, connexin 43; EMD, N-[2-methyl-4,5-bis(methylsulphonyl)-benzoyl]-guanidine, hydrochloride (EMD87580); JNK1/2 MAPK, c-Jun N-terminal kinase; KB-R7943, 2-[2-[4-(4-Nitrobenzyl)oxy]phenyl]ethyl]isothiourea mesylate; Lipo, lipofectamine; NHE-1, sodium-hydrogen exchanger-isoform 1; PE, phenylephrine; NCX, sodium calcium exchanger;
NRVM, neonatal rat ventricular myocytes; PD98059, 2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one; siRNA, small interfering ribonucleic acid; SB203580, 4-(4-Fluorophenyl)-2-(4-methylsulfinyl phenyl)-5-(4-pyridyl) 1H-imidazole; SN-6, 2-[4-(4-nitrobenzyloxy) benzyl] thiazolidine-4-carboxylic acid ethyl ester; SP600125, Anthra(1,9-cd)pyrazol-6(2H)-one 1,9-Pyrazoloanthrone

**Recommended Section:** Cardiovascular.
Connexin 43, the major connexin isoform in gap junctions of cardiac ventricular myocytes, undergoes changes in distribution and expression in cardiac diseases. The Na⁺-H⁺ exchanger (NHE-1), a key mediator of hypertrophy and heart failure has been shown to be localized in the cardiomyocyte gap junctional regions, however whether NHE-1 regulates gap junction proteins in the hypertrophied cardiomyocyte is not known. To address this question neonatal rat ventricular myocytes were treated with phenylephrine (PE) for 24 hours to induce hypertrophy. Increased Cx43 expression observed with PE treatment (132.4 ± 6.3 % compared to control; P<0.05) was further significantly augmented by the specific NHE-1 inhibitor EMD87580 (173.2 ± 8.7 % increase compared to control; P<0.05 vs PE), an effect which was mimicked by another NHE-1 inhibitor cariporide. PE-induced hypertrophy was associated with MAPK JNK1/2 activation whereas inhibition of JNK1/2 with either SP60015 or using siRNA significantly increased PE-induced upregulation of Cx43 protein levels. Inhibition of reverse mode Na⁺-Ca²⁺ exchange (NCX) with KB-R7943 partially reversed JNK1/2 activation (195.2 ± 21.4 % vs 143.7 ± 14.4 % with KB-R7943; P<0.05) and augmented upregulation of Cx43 protein (121.1 ± 8.3 % vs 215.9 ± 25.6 % with KB-R7943; P<0.05) in the presence of PE. Our results demonstrate that NHE-1 negatively regulates Cx43 protein expression in PE-induced cardiomyocyte hypertrophy via a JNK1/2 dependent pathway which is likely activated by reverse mode NCX activity.
Introduction

Gap junctions are plaques of intercellular channels connecting the cytoplasm of adjacent cells and providing electrical and metabolic coupling (Bevans et al., 1998). In the heart, gap junction channels, localized at the intercalated discs joining two cells, play a crucial role in allowing passage of current carrying ions and therefore propagation of action potential. Gap junctions differ in their abundance, size and location in the heart resulting in differences in their electrical conduction and other properties (Davis et al., 1994). Gap junctions are made up of several isoforms of connexin protein units with connexin 43 (Cx43) representing the major connexin in the working ventricular myocardium (Davis et al., 1994).

Changes in Cx43 distribution and expression have been reported in heart disease including infarction, hypertrophy and heart failure, particularly where there is an arrhythmic tendency (Saffitz et al., 1999). Cx43 is to a large degree located at the intercalated disc region (end-to-end junctions) of cardiac myocytes and some are present laterally. With hypertrophy, the proportion of Cx43 foci located laterally has been shown to increase relative to its abundance at the intercalated disc regions (Emdad et al., 2001). Changes in expression and distribution of Cx43 have been described in hypertrophic and failing human hearts (Dupont et al., 2001; Kostin et al., 2003; 2004), as well as in animal models of hypertrophy (Emdad et al., 2001; Formigli et al., 2003).

The Na⁺-H⁺ exchanger 1 isoform (NHE-1) is a major pH regulator in cardiomyocytes which functions by extruding protons in exchange for Na⁺ in a 1:1 stoicheiometric electroneutral relationship. NHE-1 has been found to be colocalized with Cx43 in the intercalated disc region of ventricular and atrial cells (Petrecca et al., 1999). In addition, increase in gap junction potential in mechanically stretched cells has been shown to be inhibited by NHE-1 blockade.
(Wang et al., 2000). In the present study we determined the potential role of NHE-1 in regulating Cx43 expression in cultured neonatal rat ventricular myocytes in which hypertrophy was induced by the administration of the $\alpha_1$ adrenoceptor agonist phenylephrine and, further, determined potential mechanisms underlying these effects.
Methods

Primary Neonatal Cardiac Myocytes Culture. All procedures were performed in accordance with the University of Western Ontario animal care guidelines which conform to the guidelines of the Canadian Council on Animal Care (Ottawa, Canada). Myocytes were prepared from hearts of 1-4 day old Sprague-Dawley rats as described previously (Gan et al., 2003). In brief, the ventricles were excised and cut into small pieces in Hanks’ Balanced Salt Solution (HBSS, (Invitrogen, Burlington, ON, Canada). The ventricles were then digested in 60 ml of collagenase (Worthington Biochemical Corporation, Lakewood, NJ) and the cells were centrifuged at 600 x g for 5 minutes at 4°C, resuspended in 10% FBS and 0.1 mM bromodeoxyuridine medium and preplated in tissue culture flasks for 30 and 60 min, respectively, to eliminate non-myocyte cells. Ventricular cells were then plated onto Primaria™ cell culture dishes (Becton Dickinson Labware, Mississauga, ON, Canada) at a concentration of 5 x 10^6 cells/60 mm dish. After 48 hours of culture the medium was replaced with serum free medium for additional overnight incubation after which the cells were treated as described below.

Experimental Design. To induce hypertrophy, myocytes were treated for 10 minutes or 24 hours as appropriate and noted in Results with 10 µM phenylephrine (Sigma, Oakville, ON, Canada) in the absence or presence of the following agents: the NHE-1 inhibitor EMD87580 (5 µM) (gift from Merck KGaA, Darmstadt, Germany) or cariporide (5 µM, gift of Sanofi-Aventis, Frankfurt, Germany), the JNK1/2 inhibitor SP600125, the p38 inhibitor SB203580, the ERK1/2 inhibitor PD98059 (all at 10 µM, from Calbiochem, Mississauga, ON, Canada), and the reverse mode Na⁺-Ca²⁺ exchange inhibitor KB-R7943 or SN-6 (both at 10 µM, fromTocris, Ellisville, MO). All drugs were added 30 minutes prior to addition of phenylephrine.
Measurement of Cell Surface Area. Cells were plated at an average density of $1 \times 10^6$ cells/60mm culture dish. After 24 hours of treatments the cells were visualized using a Leica DMIL inverted microscope equipped with a Polaroid digital camera. At least 10 random photographs were taken from each plate and the surface area of a minimum of 30 random cells from each photograph was measured using SigmaScan Software (Systat, Richmond, CA). At least 30 cells were averaged from each dish/treatment and represented as one “n” value.

Western Blotting. Cells were plated at a concentration of $4 \times 10^6$ cells/60 mm culture dish. Following treatments, the cells were washed with cold PBS and scraped into 100 µl lysis buffer (50 mM TRIS-HCl, 150 mM NaCl, 1% Triton, 10% glycerol, 2 mM EDTA, 2mM EGTA, 50 mM NaF, 200 µM Na orthovanadate, 10 mM Na$_2$P$_2$O$_7$, 40 mM B-glycerophosphate). The lysates were then homogenized and centrifuged at 10000 x g for 10 minutes at 4°C. Protein quantification of the supernatants was done by using Bradford Protein Assay Kits (Bio-Rad, Mississauga, ON, Canada). Equal amounts of total protein were loaded onto 12% SDS-PAGE gels and transferred overnight onto nitrocellulose membranes (Amersham). Blocking was done in 5% dry milk for 1 hour. The primary antibodies were incubated for 2 hours and the secondary antibody for 1 hour. The signals were detected by ECL reagent (Amersham). Primary antibodies were purchased from the following suppliers: rabbit anti-Cx43 antibody (Zymed, Markham, ON, Canada), monoclonal anti-actin antibody (Chemicon, Temecula, CA), anti-phospho-JNK1/2 (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-total JNK1/2 (Sigma).

Confocal Microscopy. Neonatal rat ventricular cells were treated for 24 hours with 10 µM phenylephrine with or without 5 µM EMD87580. At the end of the treatments myocytes were fixed in 70% ETOH for 15 min and washed three times in phosphate buffered saline (PBS) at room temperature. To simultaneously detect the presence and co-localization of Cx43 and
NHE-1, cells were incubated with primary antibodies (1µg/ml) directed toward rabbit Cx43 and murine NHE-1 for 3hrs at 4°C. Cells were subsequently washed three times in 0.1% TBS-Tween and incubated with secondary anti-antibodies (1:1000 dilution) of Alexa 488 in 0.5% BSA in PBS to detect Cx43 (red) or cy3 (green) to detect NHE-1, respectively. Cover slips were mounted and cells were visualized by confocal microscopy using an Olympus Fluoview 300 laser scanning microscopy.

Transfection of Myocytes with JNK1/2 siRNA. After plating of ventricular myocytes the medium was replaced with DMEM: M199 (4:1) serum- and antibiotic-free medium. After overnight incubation myocytes were transfected with 50 pmoles of siRNA duplex specific for JNK1/2 (5’-UCA AGG AAU AGU GUG UGC AGC UUA U-3’) or control siRNA duplex (5’-UCA UAA GGU GAG UGU CGA CUG AUA U-3’) (Invitrogen, Carlsbad, CA) in lipofectamine (Invitrogen) solution. The cells were washed the following day with PBS and incubated with DMEMF12 serum free medium for at least 24 hours before initiating treatments.

Statistical Analysis. Values are represented as means ±SE. Data were analyzed by one-way ANOVA (treatment by subject design) followed by post-hoc paired t-test with Bonferoni’s correction. A p value of <0.05 was considered to be significant.
Results

Effect of NHE-1 Inhibition on Phenylephrine-Induced Changes in Cell size and Cx43

Expression. As shown in Fig. 1 treatment for 24 hours with 10 µM phenylephrine significantly increased cell surface area to 129.6 ± 3.1 % of control values (Fig. 1A). This effect was associated with an increase in Cx43 expression of 132.4 ± 6.3% of control (Fig. 1B). The hypertrophic effect of phenylephrine was completely abrogated by 5 µM of the specific NHE-1 inhibitor EMD87580 (Fig. 1A). However, Cx43 was further significantly elevated by the presence of EMD87580 to 173.2 ± 8.7 % of control values (Fig. 1B). The effect of EMD87580 was mimicked by another NHE-1 inhibitor, cariporide, strengthening our general hypothesis of NHE-1 dependent regulation of Cx43 expression in phenylephrine treated myocytes (Fig. 2). However, in contrast to EMD87580, cariporide also directly increased Cx43 expression, in addition to augmenting the effect of phenylephrine (Fig.2). Thus, attenuation of phenylephrine-induced hypertrophy by NHE-1 inhibition is associated with elevation in Cx43 expression suggesting that NHE-1 regulates Cx43 expression in hypertrophy produced by phenylephrine administration.

Effect of NHE-1 Blockade on Cx43 Protein Distribution in Phenylephrine-Treated Ventricular Myocytes. We further determined the effect of NHE-1 blockade on Cx43 distribution in the presence of phenylephrine. As shown in Fig. 3, Cx43 and NHE-1 fluorescence signals were to a substantial degree colocalized in ventricular myocytes at gap junctions although NHE-1 was also detected in the nuclei and cytoplasm. Phenylephrine increased Cx43 protein and produced a more widespread distribution appearing as punctuate structures although colocalization of NHE-1 in gap junctions was maintained. EMD87580
substantially reversed the irregular distribution of Cx43 in phenylephrine treated myocytes (Fig. 3).

**Role of MAP Kinases on Cx43 Responses.** Connexins are regulated by numerous phosphorylation dependant mechanisms mediated by different kinases including MAP kinases (Cruciani and Mikalson, 2002). We therefore next studied the potential role of MAP kinases by first treating cells with MAPK inhibitors including the p38 inhibitor SB 203580, the ERK1/2 inhibitor PD98059 or the JNK1/2 inhibitor SP600125. As shown in Fig. 4, neither p38 or ERK1/2 inhibition exerted any effect on phenylephrine-induced upregulation of Cx43 although this was significantly augmented by the JNK1/2 inhibitor SP600125 (Fig. 4A).

Previous studies have shown that stretch-induced activation of MAP kinase can be reduced by 60% following NHE-1 blockade (Yamazaki et al., 1998). To assess whether JNK1/2 activation by phenylephrine occurs downstream of NHE-1, we determined the effect of NHE-1 inhibition on activated (phosphorylated) forms of JNK1/2 induced by 10 minutes of phenylephrine treatment. As shown in Fig. 4B phenylephrine significantly increased JNK1/2 phosphorylation which was completely prevented by EMD87580, the latter suggesting that phenylephrine-induced JNK1/2 stimulation is dependent on NHE-1 activity.

**Possible Role of Reverse Mode of NCX Activity in Phenylephrine-Induced JNK1/2 Activation.** It is known that a possible consequence of NHE-1 activation is elevation in intracellular Na\(^+\) levels leading to elevation in Ca\(^{2+}\) in cardiomyocytes through reverse mode NCX activity (Perez et al., 2001). We hypothesized therefore that the ability of phenylephrine to stimulate JNK1/2 activation may reflect secondary reverse mode NCX activation. Indeed, as shown in Fig. 5A, KB-R7943 significantly attenuated the ability of phenylephrine to activate JNK1/2 from 195.2 ± 21.4% of control values to 143.7± 14.4% in the absence or presence of
KB-R7943, respectively (P<0.05), a finding which supports the role of NCX as a factor contributing to JNK1/2 activation in phenylephrine-treated myocytes. Moreover, KB-R7943 significantly (P<0.05) augmented the increased Cx43 protein expression in myocytes exposed to phenylephrine from 121.1 ± 8.3 % to 215.9 ± 25.6 % (Fig. 5B). These effects were associated inhibition of phenylephrine-induced hypertrophy by KB-R7943 (Fig. 6). Virtually identical results were obtained with the use of another reverse mode NCX inhibitor, SN-6 in terms of JNK activation and Cx43 expression (Fig.7) and inhibition of phenylephrine-induced hypertrophy (not shown). When taken together, these data suggest that phenylephrine-induced JNK1/2 activation occurs via a reverse mode NCX-dependent pathway which in turn suppresses Cx43 protein expression.

**Effect of JNK1/2 Silencing on Cx43 Protein Levels in the Presence of Phenylephrine.** To further demonstrate the role of JNK1/2 in mediating downregulation of Cx43 protein, we used siRNA to silence JNK1/2 expression. As shown in Fig. 8A and 8B, 50 pmoles of JNK1/2 siRNA suppressed the total JNK1/2 gene expression to 12.9 ± 4.4% and protein levels to 64.1 ± 0.9% of control values (P<0.05 vs siRNA-control). Higher concentrations of siRNA were not attempted because of substantial direct effect of the transfer agent lipofectamine on cellular morphology. Therefore this concentration of siRNA was then selected to determine its effect on Cx43 expression in cultured myocytes. As shown in Fig. 9, phenylephrine in the presence of lipofectamine tended to upregulate Cx43 to 146.6 ± 28.9 % of control values although this was found to be not significantly different from control, the latter likely reflecting a direct influence of lipofectamine on phenyephrine-induced Cx43 upregulation. However cells transfected with 50 pmoles of JNK1/2 siRNA demonstrated a three-fold elevation in Cx43 protein levels which was not further augmented by phenylephrine. The ability of siRNA targeting JNK1/2 to produce
a large increase in Cx43 protein expression strongly implicates endogenous JNK1/2 as an important negative modulator of Cx43 protein expression.
Discussion

This present study demonstrates for the first time that NHE-1 is an endogenous regulator of Cx43 protein expression in hypertrophic cardiomyocytes subjected to treatment with the $\alpha_1$ adrenoceptor agonist phenylephrine. This conclusion is based on two primary observations. First, we show that NHE-1 and Cx43 are to a large degree colocalized in cultured ventricular myocytes. Second, inhibition of NHE-1 activity in myocytes markedly increases Cx43 expression in phenylephrine-treated cells concomitant with an abrogation of the hypertrophic response. The upregulation of Cx43 expression by hypertrophic stimuli using an identical cell culture preparation has been previously demonstrated for both endothelin-1 and angiotensin II (Polontchouk et al., 2002) suggesting that this effect represents a general compensatory response to hypertrophy. The ability of NHE-1 inhibition to augment the increase in Cx43 expression while at the same time abrogating the hypertrophic response to phenylephrine shows that the elevation in Cx43 expression can be dissociated from hypertrophy per se. In addition, this finding is compatible with the notion of a beneficial effect of NHE-1 inhibition in attenuating the hypertrophic and remodeling responses to pathological insult since early upregulation of Cx43 expression is meant to provide the heart with improved electrical conduction and cardiac function during pathological conditions (Saffitz, 2000). Thus, in addition to the established salutary effects of NHE-1 inhibition in attenuating hypertrophy and remodelling (reviewed in Cingolani and Ennis, 2007; Karmazyn et al., 2008) these results suggest that additional benefit may lie in the ability of these agents to increase Cx43 expression thus potentially improving cardiac functioning in parallel with reduced hypertrophy.

It should be noted that the effect of NHE-1 inhibition on Cx43 may not be restricted to quantitative changes but also to producing a more favourable cellular distribution pattern of Cx43 after phenylephrine addition since the organization/distribution of Cx43 protein in the
working ventricle is crucial for normal and effective cardiac function. Cx43 protein is mainly concentrated at the junction of two neighboring cells facilitating passage of electrical impulses for enhanced and synchronous contraction. During hypertrophy distribution of Cx43 assumes a lateral distribution leading to increased anisotropy (Emdad et al., 2001). This gap junctional remodeling results in disruption of orderly arrayed intercellular electrical conduction (Cooklin et al., 1998). In our study, phenylephrine treatment resulted in increased levels of Cx43 as determined by Western blotting although punctate distribution of the protein was observed using confocal imaging. Such discontinuous distribution patterns for Cx43 in response to phenylephrine suggests a possible basis for aberrant cell-cell communication in hypertrophy and therefore an attenuation of this response by EMD may represent one of the factors contributing to the beneficial effects afforded by NHE-1 inhibition against myocardial remodelling.

**Role of JNK1/2 as an Endogenous Regulator of Cx43**

Based on the fact that the MAP kinase pathway is important in mediating hypertrophy induced by α1 adrenergic receptor activation (Lazou et al., 1998) and that various connexins are regulated by numerous phosphorylation dependant mechanisms including MAP kinases (Cruciani asnd Mikelson, 2002) we focussed on the potential role of the latter in order to gain cellular mechanistic insights into the regulation of Cx43 in the hypertrophied cardiomyocyte. Our study demonstrated that phenylephrine-induced upregulation of Cx43 expression was associated with a significant activation of JNK1/2. Interestingly, however, the ability of the NHE-1 inhibitor EMD87580 to augment phenylephrine-induced Cx43 expression was associated with an abrogation of JNK1/2 activation. Moreover, inhibition of JNK1/2 activation with SP600125 resulted in further upregulation of Cx43 protein levels to a similar extent as that observed with NHE-1 blockade. Taken together, these findings are strongly suggestive of a role
of JNK1/2 as an endogenous regulator of Cx43 expression. This concept is further strengthened by the finding that inhibiting JNK1/2 using siRNA was associated with significantly enhanced Cx43 expression levels which was not further increased by phenylephrine treatment. Our findings are in concert with a previous report showing downregulation of Cx43 at protein and mRNA levels in cardiomyocytes infected with a JNK1/2-specific upstream activator or in transgenic mice with targeted activation of JNK1/2 in ventricular myocardium (Petrich et al., 2002). However, the amount of reduction in Cx43 protein content was relatively greater than the reduction in mRNA suggesting that activation of JNK1/2 also regulates Cx43 expression at a post transcriptional level. Our results are in partial agreement with a very recent report demonstrating increased Cx43 expression and MAPK (ERK, p38 and JNK1/2) phosphorylation in neonatal rat ventricular myocytes treated with phenylephrine (Salameh et al., 2008). These investigators demonstrated that both ERK and p38 inhibition abolished phenylephrine-induced upregulation in Cx43 although the effect of JNK1/2 inhibition or the hypertrophic responses to phenylephrine were not reported. However, our studies differ from this report as we failed to observe any effects of either ERK or p38 inhibition on Cx43 expression. The reasons for this apparent discrepant finding are uncertain at present and require further studies. However, when taken together it is possible that MAPKs play complex roles in the regulation of Cx43, especially during hypertrophy.

**Potential Role of Reverse Mode Na⁺-Ca²⁺ Exchanger in Regulating Cx43 Protein Expression**

Increased activity/expression of NHE-1 during hypertrophy/heart failure leads to increase in intracellular Na⁺ concentrations in exchange for H⁺ ion extrusion (Baartscheer et al., 2003) leading to elevation of calcium ions inside the cell (Murphy et al., 1999; Perez et al., 2001). Elevated Ca²⁺ levels activate MAP kinase pathways including JNK1/2 (McDonough et
al., 1997) and Ca\(^{2+}\) has been shown to act as a growth promoting signal (Marban and Koretsune, 1990). In the present study, activation of JNK1/2 by phenylephrine was reversed significantly by the reverse mode NCX inhibitor KB-R7943. Also, KB-R7943 in the presence of phenylephrine resulted in a further increase in Cx43 expression compared to that seen with phenylephrine alone. The effects seen with KB-R7943 on both JNK1/2 activation and Cx43 expression were reproduced by another reverse mode NCX inhibitor, SN-6. The results therefore suggest that both increased Ca\(^{2+}\) or JNK1/2 activation negatively regulates Cx43 protein expression. Based on our results, we propose the following mechanism underlying NHE-1-dependent modulation of Cx43 expression in phenylephrine-treated cardiomyocytes (Fig. 10). In this scenario phenylephrine-induced Cx43 upregulation is countered by increased NHE-1 expression which results in elevation in intracellular Na\(^{+}\) concentrations driving reverse mode NCX activity. The latter results in increased JNK1/2 activation, most likely via an elevation in intracellular Ca\(^{2+}\) concentrations thereby producing a JNK1/2-dependent attenuation of Cx43 expression through yet to be determined mechanisms although possibly though transcriptional modulation. From a pharmacological perspective, this hypothesis is supported by the fact that both NHE-1 and reverse mode NCX inhibition resulted in an attenuation of phenylephrine-induced JNK1/2 activation and a further increase in Cx43 expression, effects which were shared by JNK1/2 inhibition with SP600125. In addition, as was observed with NHE-1 inhibitors, reverse mode NCX inhibition also prevented phenylephrine-induced cardiomyocyte hypertrophy. It could therefore be postulated that the entry of Ca\(^{2+}\) via reverse mode NCX secondary to NHE-1 activation represents a major contributor to hypertrophy as well as reduced Cx43 expression due to JNK1/2 activation (Fig. 10).
To further implicate JNK1/2 in Cx43 regulation we used JNK1/2 specific siRNA to reduce JNK1/2 protein levels by a maximum of approximately 40% with 50 pmoles siRNA. Higher concentrations were not possible due to direct effects of lipofectamine on cardiomyocytes. Indeed, even at the concentration used the presence of the lipofectamine solution substantially blunted phenylephrine-induced Cx43 upregulation. Nonetheless, downregulation of JNK1/2 with siRNA markedly increased Cx43 expression further enhancing the concept of JNK1/2-dependent Cx43 regulation.

**Conclusion and Clinical Relevance**

In conclusion, our study suggests that NHE-1 activity attenuates Cx43 upregulation in hypertrophic cardiomyocytes exposed to phenylephrine which is reversed by NHE-1 inhibition. NHE-1 appears to suppress Cx43 synthesis via a pathway involving JNK1/2 activation secondary to stimulation of reverse mode NCX activity. These findings offer novel evidence for involvement of NHE-1 in mediating cardiac dysfunction associated with hypertrophic remodelling by depressing Cx43 upregulation and offers a mechanistic basis for the salutary effects of NHE-1 inhibition in the remodelled myocardium acting via a JNK1/2 and reverse mode NCX pathway. Although results obtained using cultured myocytes should be interpreted cautiously, the upregulation of Cx43 expression in response to phenylephrine may be analogous to the reported increased left ventricular Cx43 expression which has been shown to take place in patients during the compensatory phase of heart failure and which was diminished during decompensation (Kostin et al., 2004). Furthermore, it is attractive to speculate that the ability of NHE-1 inhibitors to upregulate Cx43 levels contributes to the overall favourable effects of these drugs seen in experimental heart failure (Cingolani and Ennis, 2007; Karmazyn et al., 2008).
References


Footnotes

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Legends for Figures:

**Fig. 1.** Effect of NHE-1 inhibition with 5 µM EMD 87580 on cardiomyocyte cell size (A, n=6) and Cx43 protein expression (B, n=9) in cultured neonatal rat ventricular myocytes treated for 24 hours with 10 µM phenylephrine (PE). Bars indicate means ± S.E.M (n=6 per group). *P<0.05 vs control; #P<0.05 vs PE. Top panel depicts representative micrographs showing cell characteristics after various treatments (horizontal bar = 50 µm). Representative Western blots are shown in bottom panels (B).

**Fig 2.** Effect of NHE-1 inhibition with 5 µM cariporide on cardiomyocyte cell size (A, n=14) and Cx43 protein expression (B, n=14) in cultured neonatal rat ventricular myocytes treated for 24 hours with 10 µM phenylephrine (PE). Bars indicate means ± S.E.M (n=6 per group). *P<0.05 vs control; #P<0.05 vs PE. Top panel depicts representative micrographs showing cell characteristics after various treatments (horizontal bar = 50 µm). Representative Western blots are shown in bottom panels (B).

**Fig.3.** Laser scanning confocal microscopic images of cultured neonatal rat ventricular myocytes treated for 24 hours with 10 with phenylephrine (PE) (10 µM) in the presence or absence of EMD8780 (5 M) for 24 hours showing colocalization of Cx43 and NHE-1 proteins. Green staining represents NHE-1 and the red staining represents Cx43. Yellow staining (composite) represents the localization of both Cx43 and NHE-1. Bar represents 10 µm.

**Fig. 4.** Effect of MAP kinase inhibitors on Cx43 protein levels in the presence of phenylephrine (A) and effect of NHE-1 blockade on JNK1/2 MAP kinase activation induced by phenylephrine.
(B). Panel A shows changes in Cx43 protein expression compared to control after treatment of myocytes with 10 µM phenylephrine (PE) alone or phenylephrine in the presence of the p38 inhibitor SB203580, the ERK1/2 inhibitor PD98059 or the JNK1/2 inhibitor SP600125 (each at 10 µM) for 24 hours (n=7 per group). Panel B demonstrates changes in p-JNK1/2 levels in response to phenylephrine (PE, 10 µM) and/or EMD87580 (5 µM) treatment for 10 minutes. Values represent mean ± S.E.M (n=5 per group). *P<0.05 vs control; #P<0.05 vs phenylephrine. Representative Western blots are shown in bottom panels.

**Fig. 5.** Effect of the reverse mode Na⁺-Ca²⁺ exchange inhibitor KB-R7943 (10 µM) on p-JNK1/2 (A) and Cx43 (B) expression in response to phenylephrine (PE). Phenylephrine (10 µM) and/or KB-R7943 were administered for 10 minutes for the p-JNK1/2 study and for 24 hours for Cx43 determination. Values represent mean ± S.E.M (n=5 per group). *P<0.05 vs control; #P<0.05 vs phenylephrine. Representative Western blots are shown in bottom panels.

**Fig. 6.** Effect of the reverse mode Na⁺-Ca²⁺ exchange inhibitor KB-R7943 (10 µM) on cardiomyocyte cell size in cultured neonatal rat ventricular myocytes treated for 24 hours with 10 µM phenylephrine (PE). Bars indicate means ± S.E.M (n=6 per group). *P<0.05 vs control; #P<0.05 vs PE. Top panel depicts representative micrographs showing cell characteristics after various treatments (horizontal bar = 50 µm).

**Fig. 7.** Effect of the reverse mode Na⁺-Ca²⁺ exchange inhibitor SN-6 (10 µM) on p-JNK1/2 (A) and Cx43 (B) expression in response to phenylephrine (PE). Phenylephrine (10 µM) and/or SN-6 were administered for 10 minutes for the p-JNK1/2 study and for 24 hours for Cx43
determination. Values represent mean ± S.E.M (n=5 per group). *P<0.05 vs control; #P<0.05 vs phenylephrine. Representative Western blots are shown in bottom panels.

**Fig. 8.** Effect of JNK1/2 silencing with siRNA on JNK1/2 protein (A) and gene (B) expression levels in the presence of phenylephrine. Cells were treated with either control or JNK1/2 specific siRNA for 24 hours. Values represent mean ± S.E.M (n=5 per group). *P<0.05 vs control. Representative Western blots are shown in bottom panels on left.

**Fig. 9.** Effect of JNK1/2 silencing with siRNA in the presence or absence of phenylephrine (PE) on Cx43 protein expression levels. All cells were treated for 24 hours. Values represent mean ± S.E.M (n=7 or 8 per group). *P<0.05 vs values obtained with phenylephrine (PE) plus lipofectamine (Lipo). Representative Western blots are shown in bottom panels.

**Fig. 10.** Proposed mechanism of regulation of Cx43 by NHE-1 in phenylephrine induced cardiomyocyte hypertrophy. See Discussion for details.
Figure 1

**A**

Cardiomyocyte Surface Area (µm²)

- Control
- EMD87580
- PE
- PE+EMD87580

**B**

Cx43 Expression/Actin (percent of control)

- Control
- EMD 87580
- PE
- PE+EMD 87580

**Figure 1**
Figure 2

A

Cardiomyocyte Surface Area (µm²)

Control  Cariporide  PE  PE+Cariporide

0  500  1000  1500

B

Cx43 Expression/Actin (percent of control)

Control  Cariporide  PE  PE+Cariporide

0  500  1000  1500

*  #

Cx43

Actin

*  *  #
Figure 3

Control

PE

PE+EMD87580

Cx43

NHE1

Cx43 + NHE1
Figure 4
Figure 5

Figure 5 shows the expression levels of P-JNK1/2 and JNK1/2, as well as Cx43, under different conditions.

A) P-JNK1/2 and JNK1/2 expression levels under Control, KB-R7943, PE, and PE+KB-R7943 conditions.

B) Cx43 expression levels under Control, PE, and PE+KB-R7943 conditions.
Figure 6

Cardiomyocyte Surface Area (µm²)

- Control
- KB-R7943
- PE
- PE+KB-R7943

Legend:
- Control
- KB-R7943
- PE
- PE+KB-R7943

* Indicates significant difference from control.
# Indicates significant difference from PE treatment.
Figure 7
Figure 8
Figure 9

Cx43 Expression/ GAPDH (percent of control)

- PE + Lipo
- JNK siRNA
- PE + JNK siRNA

*
Figure 10