Sodium Hydrogen Exchange 1 (NHE-1) Regulates Connexin 43 Expression in Cardiomyocytes via Reverse Mode Sodium Calcium Exchange and c-Jun NH₂-Terminal Kinase-Dependent Pathways

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ABSTRACT

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 h 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 [N-[2-methyl-4,5-bis(methylsulfonyl)-benzoyl]-guanidine methanesulfonate], PE-induced hypertrophy was associated with mitogen-activated protein kinase c-Jun NH²-terminal kinase (JNK) 1/2 activation, whereas inhibition of JNK1/2 with either SP600125 [anthra(1,9-cd)pyrazol-6(2H)-one 1,9-pyrazoloanthrone] or small interfering RNA significantly increased PE-induced up-regulation of Cx43 protein levels. Inhibition of reverse mode Na⁺-Ca²⁺ exchange (NCX) with KB-R7943 [2-[2-[4-(4-nitrobenzoxoxy)phenyl]ethyl]isothiourea mesylate] partially reversed JNK1/2 activation (195.2 ± 21.4 versus 143.7 ± 14.4% with KB-R7943; P < 0.05) and augmented up-regulation of Cx43 protein (121.1 ± 8.3 versus 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 probably activated by reverse mode NCX activity.

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

ABBREVIATIONS: Cx43, connexin 43; EMD87580, N-[2-methyl-4,5-bis(methylsulfonyl)-benzoyl]-guanidine hydrochloride; JNK, c-Jun NH²-terminal kinase; KB-R7943, 2-[2-[4-(4-nitrobenzoxoxy)phenyl]ethyl]isothiourea mesylate; NHE-1, sodium-hydrogen exchanger-isofrom 1; PE, phenylephrine; NCX, sodium calcium exchanger; PD98059, 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one; siRNA, small interfering RNA; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinyl phenyl)-5-(4-pyridyl)-1H-imidazole; SN-6, 2-[4-(4-nitrobenzoxoxy)benzyl][thiazolidine-4-carboxylic acid ethyl ester; SP600125, anthra(1,9-cd)pyrazol-6(2H)-one 1,9-pyrazoloanthrone; ERK, extracellular signal-regulated kinase; PBS, phosphate-buffered saline; MAPK, mitogen-activated protein kinase.
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; Formigl et al., 2003).

The Na$^+$/H$^+$ exchanger 1 isoform (NHE-1) is a major pH regulator in cardiomyocytes that functions by extruding protons in exchange for Na$^+$ in a 1:1 stoichiometric 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 α1-adrenoceptor agonist phenylephrine and further determined potential mechanisms underlying these effects.

Materials and 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, ON, Canada). Myocytes were prepared from hearts of 1 to 4-day-old Sprague-Dawley rats as described previously (C&an et al., 2003). In brief, the ventricles were excised and cut into small pieces in Hank’s balanced salt solution (Invitrogen, Carlsbad, CA). The ventricles were then digested in 60 ml of collagenase (Workington Biochemical Corporation, Lakewood, NJ), and the cells were centrifuged at 600g for 5 min at 4°C, resuspended in 10% fetal bovine serum and 0.1 mM bromodeoxyuridine (Sigma-Aldrich). The ventricles were then digested in Hanks’ balanced salt solution, preplated in tissue culture flasks for 30 and 60 min, and incubated with 10% fetal bovine serum and 0.1 mM bromodeoxyuridine medium. After overnight incubation, myocytes were transfected with 50 μl of the specific NHE-1 inhibitor EMD87580 (5 μM) (a gift from Merck KGaA, Darmstadt, Germany) or 4-isopropyl-3-(methylsulfonyl)benzoyl-guanidine methanesulphonate (cariporide, 5 μM) (a gift from Merck KGaA, Darmstadt, Germany).

Western Blotting. Cells were plated at a concentration of 4 × 10⁶ cells/60-mm culture dish. After treatments, the cells were washed with ice-cold phosphate-buffered saline (PBS) and scraped into 100 μl of lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 2 mM EDTA, 2 mM EGTA, 50 mM NaF, 200 μM sodium orthovanadate, 10 mM Na₃P₂O₇, 40 mM β-glycerophosphate). The lysates were then homogenized and centrifuged at 10,000g for 10 min 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-polyacrylamide gel electrophoresis gels and transferred overnight onto nitrocellulose membranes (GE Healthcare, Chalfont St Giles, UK). Blocking was done in 5% dry milk for 1 h. The primary antibodies were incubated for 2 h, and the secondary antibodies were incubated for 1 h. The signals were detected by ECL reagent (GE Healthcare). Primary antibodies were purchased from the following suppliers: rabbit anti-Cx43 antibody (Zymed, Markham, ON, Canada), monoclonal antiactin antibody (Millipore Bioscience Research Reagents, Temecula, CA), anti-phospho-JNK1/2 (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-total JNK1/2 (Sigma-Aldrich).

Results

Effect of NHE-1 Inhibition on Phenylephrine-Induced Changes in Cell Size and Cx43 Expression. As shown in Fig. 1, treatment for 24 h with 10 μM phenylephrine without or with 5 μM EMD87580. At the end of the treatments, myocytes were fixed in 70% EtOH for 15 min and washed three times in PBS at room temperature. To simultaneously detect the presence and colocalization of Cx43 and NHE-1, cells were incubated with primary antibodies (1 μg/ml) directed toward rabbit Cx43 and murine NHE-1 for 3 h at 4°C. Cells were subsequently washed three times in 0.1% Tris-buffered saline-Tween 20 and incubated with secondary antiacti antibodies (1:1000 dilution) of Alexa Fluor 488 in 0.5% bovine serum albumin in PBS to detect Cx43 (red) or cy3 (green) to detect NHE-1, respectively. Coverslips were mounted, and cells were visualized by confocal microscopy using an Olympus Fluoview 300 laser scanning microscope.

Transfection of Myocytes with JNK1/2 siRNA. After plating of ventricular myocytes, the medium was replaced with Dulbecco’s modified Eagle’s medium/M199 (4:1) serum- and antibiotic-free medium. After overnight incubation, myocytes were transfected with 50 pmol of siRNA duplex specific for JNK1/2 (5’-UCA AGG AUA AGU GUG UGC AGG UUA U-3’) or control siRNA duplex (5’-UCA UAA GGU GAG UGU CGA CUG AUU U-3’) (Invitrogen) in Lipofectamine (Invitrogen) solution. The cells were washed the next day with PBS and incubated with Dulbecco’s modified Eagle’s medium/F12 serum-free medium for at least 24 h before initiating treatments.

Statistical Analysis. Values are represented as means ± S.E. Data were analyzed by one-way analysis of variance (treatment by subject design) followed by post hoc paired t test with Bonferroni’s correction. A P value of <0.05 was considered to be significant.
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 punctate 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 MAPK on Cx43 Responses.** Connexins are regulated by numerous phosphorylation-dependent mechanisms mediated by different kinases, including MAPKs (Cruciani and Mikalsen, 2002). Therefore, we next studied the potential role of MAPKs by first treating cells with MAPK inhibitors, including the p38 inhibitor SB203580, the ERK1/2
inhibitor PD98059, or the JNK1/2 inhibitor SP600125. As shown in Fig. 4, neither p38 nor ERK1/2 inhibition exerted any effect on phenylephrine-induced up-regulation of Cx43, although this was significantly augmented by the JNK1/2 inhibitor SP600125 (Fig. 4A).

Previous studies have shown that stretch-induced activation of MAPK 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 min 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\(^{+}\)/H\(^{+}\) levels, leading to elevation in Ca\(^{2+}\) levels in cardiomyocytes through reverse mode NCX activity (Perez et al., 2001). Therefore, we hypothesized 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 that 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 with 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 (data 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 down-regulation of Cx43 protein, we used siRNA to silence JNK1/2 expression. As shown in Fig. 8, A and B, 50 pmol 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 versus 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 up-regulate Cx43 to 146.6 ± 28.9% of control values, although this was found to be not significantly different from control, the latter probably reflecting a direct influence of Lipofectamine on phenylephrine-induced Cx43 up-regulation. However, cells transfected with 50 pmol of JNK1/2 siRNA demonstrated a 3-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 α1-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 up-regulation 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, given that early up-regulation 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 remodeling (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 favorable cellular distribution pattern of Cx43 after phenylephrine addition, given that 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 becomes more lateral 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 suggest a possible basis for aberrant cell-cell communication in hypertrophy, and therefore, an attenuation of this response by EMD87580 may represent one of the factors contributing to the beneficial effects afforded by NHE-1 inhibition against myocardial remodeling.

Role of JNK1/2 As an Endogenous Regulator of Cx43.

Based on the fact that the MAPK pathway is important in mediating hypertrophy induced by $\alpha_1$-adrenergic receptor activation (Lazou et al., 1998) and that various connexins are regulated by numerous phosphorylation-dependent mechanisms, including MAPKs (Cruciani and Mikalsen, 2002), we focused on the potential role of the latter to gain cellular mechanistic insights into the regulation of Cx43 in the hypertrophied cardiomyocyte. Our study demonstrated that phenylephrine-induced up-regulation of Cx43 expression was associated with a significant activation of JNK1/2. However, it is interesting that 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 up-regulation 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 down-regulation 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 myocardiun (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 up-regulation 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 because 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$^+$-Ca$^{2+}$ 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 Ca$^{2+}$ levels activate MAPK 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. In addition, 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. Therefore, the results 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 Ca$^{2+}$ up-regulation 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 probably via an elevation in intracellular Ca$^{2+}$ concentrations, thereby producing a JNK1/2-dependent attenuation of Cx43 expression through yet to be determined mechanisms but possibly through tran-
expression of Cx43, further enhancing the effect of phenylephrine-induced JNK1/2 activation and a further increase in Cx43 expression, effects that 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. Therefore, it could 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 pmol of 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 up-regulation. Nonetheless, down-regulation of JNK1/2 with siRNA markedly increased Cx43 expression, further enhancing the concept of JNK1/2-dependent Cx43 regulation.

Conclusion and Clinical Relevance

Discussed 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 that 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. Therefore, it could 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).

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

**References**


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