**Connexin 43 Downregulation and Dephosphorylation in Nonischemic Heart Failure Is Associated With Enhanced Colocalized Protein Phosphatase Type 2A**

Xun Ai, Steven M. Pogwizd

**Abstract**—In nonischemic heart failure (HF), ventricular tachycardia initiates by a nonreentrant mechanism, but there is altered conduction (that could lead to re-entry) that could arise from changes in gap junctional proteins, especially connexin43 (Cx43). We studied Cx43 expression and phosphorylation state in the left ventricle (LV) from an arrhythmogenic rabbit model of nonischemic HF and from patients with HF attributable to idiopathic dilated cardiomyopathy. We also investigated the role of protein phosphatases that dephosphorylate Cx43—PP1 and PP2A. In HF rabbit LV, Cx43 mRNA and total protein were decreased by 29% and 34%, respectively (P<0.05 and P<0.001). In controls, Cx43 was primarily in the phosphorylated state, but with HF there was a 64% increase in nonphosphorylated Cx43 (Cx43-NP, normalized to total Cx43; P<0.05). Similar results were noted in HF rabbit myocytes (P<0.05) and in human idiopathic dilated cardiomyopathy LV (P<0.05). We found that PP1 and PP2A colocalized with Cx43 in rabbit LV. With HF, the level of colocalized PP2A increased >2.5-fold (P<0.002), whereas colocalized PP1 was unchanged. We also found intercellular coupling (assessed by Lucifer Yellow dye transfer) was markedly reduced in HF. However, okadaic acid (10 nmol/L) reduced the amount of Cx43-NP and significantly improved cell coupling in HF. Thus, in nonischemic HF in rabbits and humans, there is a decrease in both Cx43 expression and phosphorylation that contributes to uncoupling. Increased levels of PP2A that colocalize with Cx43 can underlie enhanced levels of Cx43-NP in HF. Modulation of Cx43 phosphorylation may be a potential therapeutic target to improve conduction in HF. *(Circ Res. 2005;96:54-63.)*

**Key Words:** gap junctions ■ phosphorylation ■ phosphatases ■ heart failure ■ arrhythmia

Heart failure (HF), whether nonischemic or ischemic, is associated with a nearly 50% incidence of sudden death, primarily from ventricular tachycardia (VT) degenerating to ventricular fibrillation (VF).¹ Whereas VT in nonischemic HF initiates primarily by a nonreentrant mechanism,² myocardium from patients with idiopathic dilated cardiomyopathy (IDCM) exhibits nonuniform anisotropy, slow conduction, and conduction block³ that could underlie reentry during the transition from VT to VF. Conduction slowing could arise from decreased depolarizing currents and/or decreased gap junctional coupling.⁴ However, the degree of slow conduction and block in failing myocardium appears to be out of proportion to the changes in active membrane properties.⁵ Moreover, LV myocytes from an animal model of nonischemic HF exhibit markedly decreased gap junctional conductance.⁶ Thus, alterations in intercellular coupling involving cardiac gap junctions may underlie slow conduction in nonischemic HF.  

Gap junctions are specialized membrane structures consisting of arrays of intercellular channels that directly connect adjacent cells by providing chemical and electrical communication. They are composed of connexins, a multigene family of conserved proteins. The relative amounts, composition, and distribution of these connexins appear to influence the conduction properties of cells.⁷,⁸ Connexin43 (Cx43) is the major connexin protein in ventricular myocardium, and downregulation of Cx43 (mRNA and protein) has been demonstrated in some experimental HF models and in the failing human heart.⁹ Studies in Cx43 heterozygous knockout mice, and in the canine rapid pacing HF model, support the fact that decreased Cx43 expression per se can lead to a slowing of conduction, and that arrhythmogenesis can be further enhanced under pathophysiologic conditions (eg, myocardial ischemia) and when gap junctional alterations are heterogeneous.¹⁰–¹² 

Cx43 is a phosphoprotein that can be phosphorylated by a number of kinases¹³ and dephosphorylated by protein phosphatases such as PP1 and PP2A.¹⁴ Most phosphorylation sites are serine residues on the C-terminus, although threonine and tyrosine phosphorylation sites are present to a much lesser

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Original received April 22, 2003; resubmission received February 20, 2004; revised resubmission received November 15, 2004; accepted November 22, 2004.

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*Circulation Research* is available at http://www.circresaha.org

DOI: 10.1161/01.RES.0000152325.07495.5a
extent.\textsuperscript{13} Whereas the Cx43 phosphorylation states mediated by kinases can have variable effects on gap junctional communication,\textsuperscript{15,16} dephosphorylation of Cx43 (by phosphatases) has been shown to decrease gap junctional communication—whether assessed in neonatal rat ventricular cell pairs with activation of endogenous phosphatases\textsuperscript{14} or in perfused whole rat hearts during myocardial ischemia.\textsuperscript{17} Little is known about whether there is altered Cx43 phosphorylation in HF; however, if so, it could contribute to altered conduc-
tion and arrhythmogenesis.\textsuperscript{18}

Total cellular PP1 and PP2A activity is increased in the failing heart.\textsuperscript{19} Recent studies have shown that Cx43 interacts with a number of proteins (eg, ZO-1 and protein kinase C) to form a protein complex.\textsuperscript{15,20} It is conceivable that protein phosphatases may also directly associate with Cx43 at gap
junctions to modulate its phosphorylation state, as has recently been shown for other macromolecular complexes,\textsuperscript{21} and that the level of protein phosphatases in direct proximity to Cx43 may be enhanced in HF.

As such, the goals of these studies were to investigate: (1) alteration of Cx43 protein and mRNA expression; (2) change in Cx43 phosphorylation state; (3) possible colocalization of PP1 and PP2A with Cx43; (4) alteration of intercellular coupling; and (5) the contribution of PP1 and PP2A to Cx43 dephosphorylation and altered cell coupling in HF. Studies were performed in LV tissue and myocytes from an arrhyth-
mogenic rabbit model of nonischemic HF in which 90\% of HF rabbits exhibit spontaneous VT and 10\% die suddenly.\textsuperscript{2,22} These were validated by studies in LV tissue from patients with end-stage HF attributable to IDCM and from nonfailing human hearts.

Materials and Methods
An expanded Materials and Methods section is in the online data supplement available at http://circres.ahajournals.org.

Arrhythmogenic Rabbit Nonischemic HF Model
New Zealand White rabbits underwent induction of HF by aortic insufficiency followed by thoracic aortic constriction, as previously described, until LV end-systolic dimension (LVESD) exceeded 1.20 cm (>30\% increase).\textsuperscript{2-22} HF and age-matched control rabbit hearts were rapidly excised and the LV free wall was flash-frozen in liquid nitrogen. LV myocytes were isolated as previously described.\textsuperscript{22} The protocol was approved by the University of Illinois at Chicago Animal Studies Committee.

Human Heart Tissue
LV tissue from failing human hearts (end-stage IDCM, n=8, mean ejection fraction 13±2\%) was obtained at the time of cardiac transplantation performed at University of Illinois at Chicago or Loyola University Hospital. LV tissue from nonfailing human hearts that were not used for transplantation for technical reasons (but with normal LV function, n=8) was obtained through the Regional Organ Bank of Illinois. Immediately after explantation, LV tissue was flash-frozen in liquid nitrogen. The study was approved by the Human Studies Committees of University of Illinois at Chicago, Loyola University, and Regional Organ Bank of Illinois.

Northern and Western Blot Analysis
High-yield undegraded mRNA and protein were used in all studies. Synthesis of RNA probes and Northern hybridization were based on methods of Srivastava et al.\textsuperscript{23} with modifications.

Western blotting was performed as previously described.\textsuperscript{22} Total Cx43 protein (Cx43-T) was assessed using a polyclonal Cx43-T antibody (Zymed). The nonphosphorylated isofrom of Cx43 (Cx43-NP) was detected by a specific monoclonal Cx43-NP antibody (Zymed).\textsuperscript{17}

Immuonoconfocal Microscopy and Quantitative Analysis
Immunolabeling was performed with polyclonal Cx43-T and mono-
clonal Cx43-NP antibodies described, monoclonal Cx43-T antibody (Chemicon), and monoclonal PP1 and PP2A catalytic subunit (PP2A\textsubscript{c}) antibodies (BD Sciences). Images were collected from a laser scanning confocal microscope (Carl Zeiss) and quantitatively analyzed using National Institutes of Health image software.

Phosphatase Digestion and Phosphatase Inhibition
LV homogenates were incubated with calf intestine alkaline phos-
phatase (Roche) for phosphatase digestion, and isolated myocytes were incubated with 10 nmol/L okadaic acid (OA; Sigma) for phosphatase inhibition. Changes in substrate phosphorylation status were analyzed by immunoblotting with Cx43-T and Cx43-NP antibodies.

Cosedimentation and Coimmunoprecipitation
Sedimentation of PP1 and PP2A from rabbit LV homogenates was performed using microcystin-Sepharose beads (Upstate). Immuno-
precipitation of Cx43-T using a specific monoclonal Cx43-T anti-ody was based on the method of Ausabel et al.\textsuperscript{24} with modifications. Immunoblotting was then performed with polyclonal and monoclonal Cx43-T antibodies and monoclonal PP1 and PP2A\textsubscript{c} antibodies as described.

Microinjection of Lucifer Yellow
Lucifer Yellow 4\% combined with Rhodamine B dextran 1\% was microinjected into one cell of each cell pair in M199 cell culture medium in the absence or presence of OA (10 nmol/L), according to the method of Ruiz-Meana et al.\textsuperscript{25} Microinjection and image recording were performed under a confocal microscope (Carl Zeiss).

Statistical Analysis
All data were presented as means±SEM. Differences between two groups were evaluated using Student t test, and P<0.05 was considered to be significant.

Results
Nonischemic HF Rabbit Echocardiographic Analysis
HF rabbit hearts (n=15) exhibited marked LV dilatation and systolic dysfunction compared with their baseline condition (as well as compared to age-matched controls, n=8; Table, see online supplement). With HF, LV end-diastolic dimension (LVEDD) increased by 46\% (P<0.001), LV end-systolic
dimension (LVESD) increased by 63\% (P<0.001), and mean fractional shortening (FS) decreased by 23\% (P<0.001). Heart weight and heart-to-body weight ratio were increased by 66\% (P<0.001) and 80\% (P<0.001), respectively, in HF versus controls, whereas body weight did not differ.

Cx43 mRNA and Protein Expression in Control and HF Rabbit LV
Cx43 mRNA was detected by Northern blotting as a single~3-kb band (Figure 1A, top), and its abundance was normalized to GAPDH mRNA (Figure 1A, bottom). There was a 29\% decrease in normalized Cx43 mRNA in HF versus
controls (n=7 and n=6, respectively; *P<0.05; Figure 1B). Similar results were found when Cx43 mRNA was normalized to 18S ribosomal mRNA (data not shown).

Western blot analysis was performed in LV homogenates from HF and age-matched control hearts, and the signal increased linearly with respect to the amount of protein loaded (Figure I in the online data supplement). The polyclonal Cx43-T antibody recognizes both phosphorylated and Cx43-NP isoforms. As such, multiple bands (corresponding to Cx43-NP and higher-molecular-weight phosphorylated isoforms of Cx43) migrated at 42 to 46 kDa. Cx43 total protein (normalized to GAPDH) was decreased 34% in HF versus controls (n=14 and n=14, respectively; *P<0.001; Figure 1D, left).

**Cx43 Phosphorylation in Isolated Rabbit Myocytes**

Immunoblot data from isolated rabbit LV myocytes revealed that Cx43-T protein expression (measured as the 42 to 46 kDa band) was decreased 30% in HF versus controls (P<0.05; n=7 and n=5, respectively; Figure 2A and 2B, left), with a similar shift from phosphorylated Cx43 to the lower-molecular-weight Cx43-NP. As mentioned, we also determined the proportion of phosphorylated to nonphosphorylated Cx43 and found that it changed from 86:14 (or 6:1) in control to 75:25 (or 3:1) with HF (Figure 2B, middle). When we used the antibody specific for Cx43-NP, we found that with HF, the level of Cx43-NP (normalized to total Cx43) increased by 69% (P<0.05; Figure 2B and 2C, right), results that were comparable to those noted in LV tissue (Figure 1D).

We confirmed these changes in Cx43 phosphorylation states with HF by immunofluorescent staining using confocal microscopy. Double staining was performed in isolated rabbit LV myocytes with Cx43-T and Cx43-NP antibodies. There was no fluorescence signal found by staining with the secondary antibody only (negative control, data not shown). Figure 2C shows that Cx43 protein was localized throughout...
the myocyte membrane in distinctive punctuate patterns, primarily at cell ends (rather than sides). There was similar localization between immunolabeled Cx43-T (green) and Cx43-NP isoform (red) signals. Cx43-T was reduced by 28% and Cx43-NP (normalized to Cx43-T protein) was enhanced by 71% in HF myocytes versus controls (P < 0.05; n=3 and n=3, respectively; Figure 2D), results that were consistent with the immunoblotting data in rabbit LV tissue and myocytes above.

**Cx43 Expression and Phosphorylation States in LV From Patients With IDCM**

To validate the results from HF rabbit LV, similar experiments were performed in LV tissue from nonfailing and failing (IDCM) human hearts. The Northern blot data revealed that Cx43 mRNA (normalized to GAPDH) was reduced by 28% in IDCM versus nonfailing human LV (n=8 and n=8, respectively; P<0.05; Figure 3A and 3B).

Immunoblotting of human LV homogenates was performed using both Cx43-T and Cx43-NP antibodies, and the results were normalized to GAPDH (Figure 3C and 3D). Cx43-T was decreased by 25% in IDCVM versus nonfailing LV (n=8 and n=8, respectively; P<0.05, left). From band analysis of the Cx43 total blot (as described), we calculated that the proportion of phosphorylated to nonphosphorylated Cx43 changed from 88:12 (or 8:1) in control to 80:20 (or 4:1) with IDCM (Figure 3D, left), and the ratio of Cx43-NP to Cx43-T increased 65% (P<0.01; Figure 3D, middle). There was a 60% increase (P<0.05; Figure 3C and Figure 3D, right) in Cx43-NP (normalized to total Cx43) in HF by using the specific Cx43-NP antibody. All these results, along with phosphatase digestion data shown in Figure 3E, were comparable to the data from HF rabbit LV described.

**Colocalization of Protein Phosphatases With Cx43 in LV and Enhanced Colocalized Protein Phosphatases With HF**

To determine whether enhanced Cx43-NP, which was observed in HF LV, is related to the interaction of protein phosphatases (PP1 and PP2A) with Cx43, coimmunoprecipitation, cosedimentation, and immunoconfocal analysis were performed in control and HF rabbit LV tissue.

To explore for a possible direct interaction of PP1 and PP2A with Cx43, PP1 and PP2A proteins were first isolated from homogenates by binding to microcystin (a specific inhibitor of PP1 and PP2A), followed by immunoblotting...
analysis. We observed a single cosedimented Cx43 reactive band at 42 to 46 kDa (Figure 4A, top), along with PP1 and PP2Ac bands (Figure 4A, middle and bottom).

We confirmed these findings by immunoprecipitation using a monoclonal Cx43-T antibody. Along with immunoprecipitated Cx43 bands (which were detected by both polyclonal and monoclonal Cx43-T antibodies, and which were distinct from IgG bands), immunoblotting images revealed coimmunoprecipitated PP1 and PP2Ac reactive bands (Figure 4B). IgG alone was the negative (data not shown). Quantitation of the coimmunoprecipitated PP1 and PP2Ac (normalized to immunoprecipitated Cx43 in each individual sample) revealed that the level of PP1 was unchanged, but the level of coimmunoprecipitated PP2Ac was increased 2.5-fold in HF rabbit LV tissue versus controls (n=11005 and n=4, respectively; *P<0.002; Figure 4C).

We further confirmed the increased colocalization of PP2Ac with Cx43 in HF by using immunofluorescence staining with Cx43-NP and PP2Ac antibodies in control and HF LV myocytes. Figure 5 shows representative confocal images where Cx43-NP isoform (red) is present in distinctive punctate patterns throughout the cell membrane, and it colocalizes with PP2Ac (green, also distributed in cytosol) that is increased 2-fold in HF myocytes (consistent with the immunoprecipitation and cosedimentation data). Similar re-

Figure 3. A, Northern blot of Cx43 mRNA (upper) and GAPDH mRNA (lower) from LV samples of nonfailing (NF) and IDCM human hearts. B, Changes in Cx43 mRNA expression with IDCM versus NF (P<0.05). C, Immunoblot images of Cx43-T (top), Cx43-NP (middle), and GAPDH (bottom) in human NF and IDCM LV. D, Summarized data for Cx43 expression in control and IDCM human LV (P<0.05, **P<0.01). At left are the changes in Cx43-T expression with the relative contributions of Cx43-NP and Cx43-P indicated. In the middle are data for the ratio of Cx43-NP isoform to Cx43-T protein (NP/Total) using the Cx43-T antibody. At right is the ratio of Cx43-NP (using a specific Cx43 phospho-antibody) to total Cx43 (using Cx43-T antibody). E, Alkaline phosphatase digestion of samples from human nonfailing and IDCM hearts.
results were found for double staining using Cx43-T and PP2Ac antibodies (data not shown). PP1 also colocalized with Cx43 at distinct membrane sites, but the amount of colocalized PP1 appeared unchanged in HF (data not shown).

Alteration of Cell–Cell Communication and the Role of PP2A in Accumulation of Dephosphorylated Cx43 in HF

To define alterations of intercellular communication in HF and its modulation by protein phosphatases, we performed Lucifer Yellow dye transfer (microinjection) in the absence or presence of the protein phosphatase inhibitor OA, at a concentration (10 nmol/L) that has been shown to inhibit PP2A, but not PP1 (Figure 6). To delineate the injected cell of the cell pairs, we also injected the high-molecular-weight (10 000) Rhodamine B dextran, which is retained in the injected myocyte without passing gap junction channels to the recipient cell (Figure 6, bottom left). Figure 6 shows images of the microelectrode and control, HF, and OA-treated HF cell pairs (injected and recipient cells), and sequential confocal images at times 1, 38, and 214 seconds. Figure 7A shows representative time course data for fluorescence (normalized to plateau) in the recipient cell (30/80 μm window; Figure 6, bottom right panel), and Figure 7B shows summarized rate constant (τ, time to reach ≈63% peak) data. HF myocytes had decreased cellular coupling, reflected by slower dye transfer with an increased τ of dye transfer of 53±12 seconds versus 19±5 seconds for control (P<0.05; n=6 and n=5, respectively). However, OA-treated HF cell pairs showed improved dye transfer with a reduced τ of dye transfer (τ=24±5; P<0.05 versus HF and P=NS versus control; n=4). Protein phosphatase inhibition

Figure 5. Double immunofluorescence staining of control (left) and HF rabbit (right) isolated LV myocytes with Cx43-NP and PP2Ac antibodies alone (top, red; middle, green), and the overlap (bottom, yellow). Scale bar is 20 μm.
by OA (10 nmol/L) was verified by immunoblotting, with OA-treated HF myocytes exhibiting a 42 ± 9.3% decrease in Cx43-NP (Figure 7C; n = 3). OA-treated control cell pairs showed no additional improvement in cell coupling (τ = 18 ± 7; n = 4; P = NS versus untreated control cell pairs) (Figure 7A).

**Discussion**

In the present study, we studied LV tissue and myocytes from an arrhythmogenic rabbit model of nonischemic HF and from age-matched control rabbits. We found that Cx43 expression was downregulated on both a protein and mRNA level. HF rabbits exhibited a significant enhancement of the nonphosphorylated isoform of Cx43 (and decreased phosphorylated Cx43). All these results were validated in LV tissue from hearts of patients with end-stage IDCM (at the time of transplantation) compared with nonfailing hearts. We also observed that protein phosphatases PP1 and PP2Ac both colocalized with Cx43 in rabbit LV, and there was a 2.5-fold increase in the level of colocalized PP2Ac in HF. Moreover, we found that intercellular coupling between LV myocytes was reduced markedly in HF. However, OA reduced the amount of Cx43-NP and significantly improved cell coupling in HF. Our observations suggest that both reduced Cx43 expression and enhanced Cx43-NP within gap junctions underlie impaired cellular coupling and ultimately altered conduction in nonischemic HF. The increased levels of colocalized PP2Ac (with Cx43) contribute to enhanced Cx43-NP in HF.

**Reduced Cx43 Expression and Increased Cx43-NP Is Associated With Altered Cell Coupling and Conduction in Nonischemic HF**

Ventricular arrhythmias (VT and VF) account for nearly half of deaths in patients with HF (both nonischemic and ischemic). In 3-dimensional cardiac mapping studies, we have previously shown that spontaneously occurring VT in nonis-
The Role of Phosphatases in Cx43 Dephosphorylation in Nonischemic HF

Reduced Cx43 phosphorylation in HF could be attributable to reduced phosphorylation by kinases (eg, protein kinase A, protein kinase C, and MAPK). However, findings in human IDCM were nearly identical. Despite the consistency of this finding, interpretation is limited because the extent to which Cx43 is phosphorylated at baseline in rabbit and human LV is not known. However, we took advantage of the Cx43 expression pattern on Western blots and determined the extent of Cx43 phosphorylation in controls and HF. Our results in controls were consistent between rabbits and nonfailing human LV. With HF, we found a significant increase in the Cx43-NP on Western blots using an extensively validated antibody specific for the Cx43-NP isoform. Under physiological conditions, the C-terminus of Cx43 is extensively phosphorylated, primarily at serine sites, although recent studies suggest there may be changes in tyrosine phosphorylation in HF. The specific Cx43-NP antibody used in the present study recognizes the nonphosphorylated serine-rich segment of the Cx43 C-terminus that includes putative phosphorylation sites for multiple kinases including protein kinase A, protein kinase C, and MAPK. Our results of Cx43-NP in HF rabbits were consistent with both LV tissue and isolated myocytes. Moreover, findings in human IDCM were nearly identical. Despite the consistency of this finding, interpretation is limited because the extent to which Cx43 is phosphorylated at baseline in rabbit and human LV is not known. However, we took advantage of the Cx43 expression pattern on Western blots and determined the extent of Cx43 phosphorylation in controls and HF. Our results in controls were consistent between rabbits and nonfailing humans (and similar to that noted by others). The changes in Cx43 phosphorylation with HF were substantive and confirmed the relative changes in Cx43-NP using a specific Cx43-NP antibody.

The present study found that HF myocytes exhibit reduced cellular coupling. This was associated with decreased Cx43 expression on both an mRNA and protein level, similar to that reported by others. Cx43 downregulation may be a common feature of failing heart, although the underlying biochemical basis remains to be determined. There has been considerable debate as to the role of Cx43 in modulating conduction,10,29 However, studies in heterozygous Cx43 knockout mice, along with those in other transgenic models, and in dogs with pacing-induced HF, support the contention that reduced Cx43 expression per se could contribute to altered cell-coupling and conduction in nonischemic HF.

Cx43 is a phosphoprotein that is predominantly phosphorylated in the control state. We confirmed this in control rabbit and nonfailing human LV. With HF, we found a significant increase in the Cx43-NP on Western blots using an extensively validated antibody specific for the Cx43-NP isoform. The exact Cx43-NP antibody used in the present study recognizes the nonphosphorylated serine-rich segment of the Cx43 C-terminus that includes putative phosphorylation sites for multiple kinases including protein kinase A, protein kinase C, and MAPK. Our results of Cx43-NP in HF rabbits were consistent with both LV tissue and isolated myocytes. Moreover, findings in human IDCM were nearly identical. Despite the consistency of this finding, interpretation is limited because the extent to which Cx43 is phosphorylated at baseline in rabbit and human LV is not known. However, we took advantage of the Cx43 expression pattern on Western blots and determined the extent of Cx43 phosphorylation in controls and HF. Our results in controls were consistent between rabbits and nonfailing humans (and similar to that noted by others). The changes in Cx43 phosphorylation with HF were substantive and confirmed the relative changes in Cx43-NP using a specific Cx43-NP antibody.

The Role of Phosphatases in Cx43 Dephosphorylation in Nonischemic HF

Reduced Cx43 phosphorylation in HF could be attributable to reduced phosphorylation by kinases (eg, protein kinase A, protein kinase C) and/or to increased dephosphorylation by protein phosphatases. Here, we focused on the role of phosphatases PP1 and PP2A, the primary phosphatases that dephosphorylate Cx43, because levels of total cellular PP1 and total cellular PP1 and PP2A activity are increased in HF. Moreover, activation of phosphatases that dephosphorylate Cx43 can reduce communication between cells.

We reasoned that local levels of an enzyme (rather than total cellular levels) may best reflect its actions, and that PP1 and PP2A might interact directly with Cx43. Cx43 is a part of a protein complex, and it has been reported that kinases and structural proteins have direct interactions with cardiac Cx43. The present study provides the first evidence of an interaction between Cx43 and PP1 and PP2Ac in rabbit LV. Cx43, PP1, and PP2A form a close complex that could be immunoprecipitated by a Cx43 antibody or sedimented by microcystin-Sepharose beads (that bind PP1 and PP2A). We confirmed this with immunofluorescence staining showing colocalization at gap junctional sites in the membrane. With HF, we found that the level of coimmunoprecipitated PP2Ac with Cx43 was dramatically increased >2.5-fold, whereas colocalized PP1 was unchanged. Moreover, OA, at a concentration that inhibits PP2A, but not PP1, and that increased the levels of phosphorylated Cx43, significantly improved cellular coupling between HF rabbit myocytes. The rapidity and near-complete recovery of coupling (even in the setting of reduced Cx43 protein levels) suggest that phosphorylation provides a dynamic means of regulation and that there may be a steep relationship between Cx43 phosphorylation state and cellular coupling (that may plateau, as reflected by the unchanged coupling in OA-treated control cell pairs). Whether dephosphorylated Cx43 per se reduces coupling remains to be determined.

Animal Model of HF

Our HF rabbit model exhibits contractile dysfunction, pathologic alterations, and arrhythmogenesis similar to nonischemic human HF and we have consistently validated biochemical and electrophysiologic findings in this model with those in nonischemic HF in humans (including the results of the present study). This suggests that this arrhythmogenic HF rabbit model is ideally suited for studies of altered Cx43 expression, phosphorylation, and function.

Limitations

Our observations strongly suggest that increased levels of colocalized PP2Ac (with Cx43) contribute to enhanced Cx43-NP and reduced cellular coupling in HF LV. Whether increased activity of other protein phosphatases, such as PP1 and calcineurin, or decreased activity of kinases contribute to Cx43 dephosphorylation in HF remains unknown.

In addition to Cx43 downregulation and altered Cx43 phosphorylation, there could be changes in gap junction distribution with HF that may modulate conduction. Although this was not assessed in the present study, recent studies have demonstrated altered gap junction distribution in human IDCM. We focused on alterations in Cx43, by far the predominant ventricular gap junction protein, so we cannot rule out alterations in the expression of other connexins (eg, Cx45 or Cx40) in HF.

Implications

Our findings in the present study suggest that the combination of Cx43 downregulation and altered phosphorylation contribute to decreased cellular coupling and, ultimately, slow conduction in nonischemic HF (Figure 8). The colocalization
of PP1 and PP2Ac with Cx43 indicates that Cx43 represents a macromolecular complex with numerous components, including both kinases and phosphatases. Enhanced levels of colocalized PP2Ac in HF indicate an additional regulation of including both kinases and phosphatases. Enhanced levels of PP1 and PP2Ac with Cx43 indicates that Cx43 represents a macromolecular complex with numerous components, including both kinases and phosphatases. Enhanced levels of colocalized PP2Ac in HF indicate an additional regulation of Cx43 phosphorylation could modulate connexin43 phosphorylation in human heart. The medical basis of anisotropy: role of gap junctions. J Cardiovasc Electrophysiol, 1995;6:498–510.


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Circ Res. 2005;96:54-63; originally published online December 2, 2004;
doi: 10.1161/01.RES.0000152325.07495.5a
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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http://circres.ahajournals.org/content/96/1/54

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Expended Materials and Methods

Arrhythmogenic rabbit nonischemic HF model

New Zealand White rabbits (~3.5 kg) underwent induction of HF by aortic insufficiency followed by thoracic aortic constriction. Progression of HF was assessed by LV end-diastolic dimension (LVEDD), LV end-systolic dimension (LVESD) and LV fractional shortening (FS) \[FS (\%) = \frac{(LVEDD-LVESD)}{LVEDD}\] using 2-D echocardiography (under sedation with ketamine 0.35mg/kg), as previously described.\(^1,2\) Animals were studied 9.5 ± 2 months later when severe heart failure developed (LVESD > 1.2 cm with LV FS ~ 25-30%). HF and age-matched control rabbits (n=14, 14) were anesthetized with isoflurane. Hearts were rapidly excised and the LV free wall was flash-frozen in liquid nitrogen.

Rabbit LV myocytes isolation

Rabbit LV myocytes were isolated as previously described.\(^2\) Hearts were mounted on a gravity-flow Langendorff perfusion apparatus (37°C) and perfused for 5 minutes with nominally Ca-free normal Tyrode’s (NT) solution (containing mmol/L: NaCl 140, KCl 4, MgCl\(_2\) 1, HEPES 5, glucose 10, pH 7.25 with NaOH). Back flow across the incompetent aortic valve in HF rabbits was blocked by a balloon-tipped catheter inflated in the LV outflow tract. Perfusion proceeded with added collagenase (0.75 mg/ml and 0.4mg/ml for control and HF, respectively) and 0.05% albumin ([Ca] = 36 µmol/L) until the heart became flaccid (20-30 minutes). LV tissue was cut into ~2 X 2 mm pieces for further incubation at 37°C in 4 ml aliquots of the same solution, but with 0.4 mg/ml
collagenase. Incubations were stopped at 1-4 minutes by adding 40 ml of 200 µmol/L Ca NT solution with 0.05% albumin, and the tissue was filtered through gauze. Cells were then washed three times with 200 µmol/L Ca NT solution (without albumin) before use.

Production of cRNA probes and Northern blot analysis:

RNA probe synthesis and Northern hybridization were based on a protocol by Srivastava et al with modifications. cDNA was prepared from total RNA of rabbit and human LV tissue. A 287-bp fragment (position 1023-1311) from human Cx43 full length mRNA sequence (NCBI, M65188) was generated as a cRNA probe template using PCR amplification at 94°C, 32°C → 54°C, 32°C → 72°C, 45°C for 30 cycles with a sense primer (5’ aagctggttactggcgacag3’) and an antisense primer (5’ggatcctaatacgactcactatagggaggtccaggtcatcaggccag3’, 23-bp T7 promoter sequence appended at the 5’ end). Screening of the NCBI gene bank indicated that this probe does not have major homology with other published sequences of cardiac connexins (Cx37, Cx40, Cx45). Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (302 bp) was synthesized by PCR amplification at 94°C, 32°C → 56°C, 32°C → 72°C, 45°C for 30 cycles with a sense primer (5’gtcagtggtggacctgacct 3’) and an antisense primer (with T7 promoter) (5’ggatcctaatacgactcactatagggaggtccaggtcatcaggccag3’, 23-bp T7 promoter sequence appended at the 5’ end). cRNA probes from human Cx43 cDNA, GAPDH cDNA and 18S antisense control templates (Ambion) were labeled with 32P-UTP by means of a Stripe-EZ kit (Ambion).

RNA blots were pre-hybridized in Rap-Hybridization buffer (Amersham) for 1hr at 68°C. Hybridization was performed by applying 2 x 10^6 cpm/ml Cx43 probe to Rap-
hybridization buffer for 2 hours at 68°C. After hybridization, the membrane was washed once with 2 x SSC + 0.1% SDS for 20 minutes at room temperature and twice with 0.1 SSC + 0.1% SDS for 20 minutes at 68°C. Autoradiography was carried out with BioMax MS film (Kodak) and densitometric quantitation was normalized to GAPDH and 18S cRNA.

Immunoblotting

Western blotting was performed as previously described.² Cx43-T protein was assessed using a polyclonal Cx43-T antibody that recognizes epitopes in the C-terminus of rat Cx43 (Zymed). The nonphosphorylated isoform of Cx43 (Cx43-NP) was detected by a specific monoclonal Cx43-NP antibody raised against a peptide consisting of residues 360-376 (DQRPSRASSRASSRPR) in the C-terminus of rat Cx43 (Zymed). The recognition site of this epitope is the non-phosphorylated residues in the serine-rich area.

Samples were homogenized in RIPA buffer with protease inhibitors (aprotinin 10 µg/ml, leupeptin 10 µg/ml, Pefabloc 1 mmol/L). Protein was quantified using a DC protein assay kit (Bio-Rad). Homogenates or cell lysates (20 µg protein) were separated by 10% SDS-PAGE gel and transferred to nitrocellulose. The transferred membrane was incubated with rabbit polyclonal Cx43-T (1:1000 dilution) or mouse monoclonal Cx43-NP antibodies (1:500) overnight at 4°C, followed by a 2-hour incubation with a peroxidase-conjugated secondary antibody. Loads were normalized by reprobing the membrane with monoclonal GAPDH antibody (1:300). Immunoreactivity was detected
by chemi-luminescence (ECL Western Blotting Analysis System, Amersham), and the intensity of the bands was quantified by densitometry.

**Phosphatase digestion and Phosphatase inhibition**

Homogenates (100 µg protein) in 100 µl Tris/MgCl₂ buffer were incubated with 30 U calf intestine alkaline phosphatase (Roche Diagnostic) overnight at 30°C. The dephosphorylation reaction was terminated by adding an equal volume of 2 X SDS-PAGE sample loading buffer. Okadaic acid was dissolved in DMSO but diluted further in buffer or media. Isolated myocytes from control and HF rabbit LV were incubated with 10nmol/L OA for 10 minutes at 37°C.

**Immunoprecipitation, co-sedimentation and immunoblotting**

Immunoprecipitation was based on the method of Ausubel et al⁴ with modifications. Homogenates (500 µg protein) of LV tissue were suspended in 0.5ml RIPA buffer with 0.25% Triton X100 and protease inhibitors. The samples were incubated with a monoclonal Cx43-T antibody overnight at 4°C (IgG alone was used as a negative control). Protein A-Sepharose beads were added with incubation at 4° C for 1 hour, followed by washing with RIPA buffer.

Sepharose beads bound with the specific PP1 and PP2A inhibitor microcystin (UBI) were used to isolate PP1 and PP2A from 500 µg of rabbit LV homogenates by incubating at 4° C for 1 hour, followed by washing in RIPA buffer. Beads were resuspended in 2x4% SDS sample loading buffer and boiled for 5 minutes. The supernatant was loaded onto a 10% SDS-PAGE gel. Immunoblots were performed to
detect Cx43 (monoclonal Cx43-T antibody, 1:1000), PP1 (monoclonal PP1 antibody, 1:1000) or PP2Ac (monoclonal PP2A catalytic subunit antibody, 1:5000) as described.³

**Immunolabeling and confocal microscopy**

Suspended isolated rabbit myocytes were attached to laminin-coated glass slides for 30 minutes, followed by fixing with 4% paraformaldehyde in PBS for 20 minutes. Cells were blocked in 10% goat serum and 0.2% bovine serum albumin with 0.2% Triton X100. For double staining with Cx43-T and Cx43-NP antibodies, myocytes were first incubated with a Cx43-NP antibody followed by an Alexa Fluor 568-conjugated secondary antibody (Molecular Probes). The cells were then pre-blocked with a rabbit IgG (H+L) antibody for 1 hour, followed by incubating with polyclonal Cx43-T antibody. After washing with PBS, cells were reacted with an Alexa Fluor 488-conjugated secondary antibody (Molecular Probes).

For double labeling with the Cx43-NP or Cx43-T and PP1 or PP2Ac antibodies, cells were incubated with monoclonal Cx43-NP or polyclonal Cx43-T antibodies, followed by an Alexa Fluor 568-conjugated or Alexa Fluor 488-conjugated secondary antibodies. The cells were then incubated either with monoclonal PP1 antibody (followed by an Alexa Fluor 350-conjugated secondary antibody) or with monoclonal PP2Ac antibody (followed by an Alexa Fluor 488-conjugated secondary antibody or Alexa Fluor 350-conjugated secondary antibody for double immunolabeling with Cx43-T antibody), and then fixed with mounting medium.

Stained cells were visualized by using a PASCAL 5 laser scanning confocal microscope (Carl Zeiss) equipped with a 63x water immersion objective. An argon laser
was used for excitation of fluorescein (488nm) and Texas red (568nm), and UV light was used for excitation of Alexa Fluor blue (351nm). Green, blue and red emissions were detected through LP 505, LP 385, and LP 585 filters, respectively. The two different fluorochromes were scanned sequentially by using a multi-tracking function to avoid any bleed-through between two different fluorescent dyes. The same detection parameters (including the size of pinhole, detection gain, amplification offset, and amplification gain) were set for collecting images from both control and HF samples. The images displayed pixel intensities between 0 and 255 gray level scales. The collected images were processed by using Adobe Photoshop software.

**Quantitative analysis of images**

The confocal images were analyzed using NIH-image (PC version, written by Wayne Rasband, NIMH, Bethesda, and developed by Sicon Corporation Inc). The pixel intensities were sampled in immunonegative regions and a threshold level was selected to allow exclusive detection and quantitation of immunostaining in positive regions. Subsequent analysis was undertaken with Macro programs written by Grant Henning.

**Microinjection and Dye transfer**

Cell pairs were cultured on coverslips for 2 hours. A hydraulic micromanipulator with digital control (digital micromanipulator model 5171 and transjector model 5246, Eppendorf picospritzer) was used to position a 21.7 MΩ resistance, 0.2-0.5-μm-tip sterile glass micro-electrode adjacent to one of the cells of the cell pair. Before injection, myocytes were incubated in the absence or presence of OA (10nmol/L) for 10 minutes
at 37°C. 4% Lucifer Yellow (excitation = 458nm; emission = 531 nm) and 1% Rhodamine B dextran (excitation = 543nm; emission = 560nm) in a buffer containing 150mmol/L LiCl and 10mmol/L Hepes were microinjected into a myocyte of an end-to-end cell pair with a pulses pressure of 400 hPa of 0.1-second duration, as described in Ruiz-Meana et al. 6 All experiments were performed at 37°C at the stage of a confocal microscope (Carl Ziess). Cell images were collected every 0.5 seconds at x32 magnifications. The extent of intercellular transfer was determined by recording fluorescence intensity in the adjacent cell of the cell pair from confocal images obtained every 12-13 seconds. 7 While these analyses were not performed in a blinded fashion, subsequent analysis performed in a blinded fashion revealed comparable results. Cell-coupling was measured as the rate of increased fluorescence in the recipient cell of the cell pair in a 30 x 80 µm window (as shown in Fig. 7 bottom right). The $\tau$ of normalized LY transfer (from a monoexponential fit) was analyzed with Prism analyzing software.

References:


### Table

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HW/BW is heart weight to body weight ratio. *p<0.001 vs control, †p<0.001 vs control and baseline. ‡ p<0.01 vs control and p<0.001 vs baseline.
Figure legend

Figure 1

Linearity of detection of the Cx43 total protein in a Western blot with Cx43-T antibody. The graph shows that the signal increases linearly with respect to the amount of protein loaded from 20µg to 60µg of homogenates from control rabbit LV. A linear regression of those results gave an R value of 0.99.