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N-cadherin haploinsufficiency affects cardiac gap junctions and arrhythmic susceptibility

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Abstract

Cardiac-specific deletion of the murine gene (*Cdh2*) encoding the cell adhesion molecule, N-cadherin, results in disassembly of the intercalated disc (ICD) structure and sudden arrhythmic death. Connexin 43 (Cx43)-containing gap junctions are significantly reduced in the heart after depleting N-cadherin, therefore we hypothesized that animals expressing half the normal levels of N-cadherin would exhibit an intermediate phenotype. We examined the effect of N-cadherin haploinsufficiency on Cx43 expression and susceptibility to induced arrhythmias in mice either wild-type or heterozygous for the *Cx43* (*Gja1*)-null allele. An increase in hypophosphorylated Cx43 accompanied by a modest decrease in total Cx43 protein levels was observed in the N-cadherin heterozygous mice. Consistent with these findings N-cadherin heterozygotes exhibited increased susceptibility to ventricular arrhythmias compared to wild-type mice. Quantitative immunofluorescence microscopy revealed a reduction in size of large Cx43-containing plaques in the N-cadherin heterozygous animals compared to wild-type. Gap junctions were further decreased in number and size in the N-cad/Cx43 compound heterozygous mice with increased arrhythmic susceptibility compared to the single mutants. The scaffold protein, ZO-1, was reduced at the ICD in N-cadherin heterozygous cardiomyocytes providing a possible explanation for the reduction in Cx43 plaque size. These data provide further support for the intimate relationship between N-cadherin and Cx43 in the heart, and suggest that germline mutations in the human *N-cadherin* (*Cdh2*) gene may predispose patients to increased risk of cardiac arrhythmias.

Keywords

arrhythmia; cell communication; cadherin; connexin; gap junction

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Introduction

The intercalated disc (ICD) contains various junctional proteins that mechanically and electrically couple cardiomyocytes ensuring normal rhythmic contractions of the four-chamber mammalian heart. Gap junctions localize to the ICD and form low resistance intercellular electrical coupling channels, many of which are extremely large, particularly with regards to mammalian myocardium. It is well appreciated that altered expression and/or distribution of Cx43-containing gap junctions are common features of diseased myocardium (1–3). Recent studies have demonstrated that cardiac-specific loss of Cx43 is sufficient to slow myocardial conduction velocity and induce unidirectional block resulting in an arrhythmogenic substrate and sudden cardiac death (SCD) in mice (4,5). However, it is less well understood how gap junctions are regulated and maintained at the ICD.

The adherens junction and desmosome mediate cell-cell coupling at the ICD via linkage to the actin cytoskeleton and intermediate filament system (i.e. desmin), respectively. The adherens junction consists of the classical cadherin, N-cadherin, and its cytoplasmic binding partners, the catenins. Desmoglein and desmocollin, also members of the cadherin superfamily, are found in the desmosome where they interact with linker proteins such as plakoglobin and desmoplakin. Recent data indicate a hybrid junctional complex exists in the heart referred to as area composita where a combination of components from the adherens junction and desmosome colocalize and interact (6–8).

Adherens junction formation is a prerequisite for gap junction assembly in cultured adult rat cardiomyocytes (9–11). Expression of a dominant negative N-cadherin in rat cardiomyocytes disrupts gap junction organization at the cell surface (9). Furthermore, gap junctions are lost in N-cadherin-null embryonic cardiomyocytes, and reassembled upon reintroduction of cadherin (12). Recent evidence indicates that N-cadherin and β -catenin control the targeting of Cx43 to adherens junctions by interacting with microtubule plus-end-tracking proteins (13). Studies in NIH3T3 cells showed that knockdown of N-cadherin affects trafficking of a N-cadherin/Cx43 multiprotein complex to the cell surface (14). Thus, N-cadherin may be involved in more than one step in the development of functionally mature gap junction plaques at the plasma membrane.

We recently demonstrated that cardiac-specific loss of N-cadherin (CKO) in mice leads to disassembly of the ICD and induces SCD (15). There is a significant reduction in Cx43-containing gap junctions in the N-cadherin CKO hearts causing decreased ventricular conduction velocity (16). In addition to N-cadherin-containing adherens junctions, desmosomes are lost from the ICD in the N-cadherin CKO hearts making it difficult to distinguish which adhesive junction is required for maintaining gap junctions at the plasma membrane. In addition, N-cadherin CKO mice have decreased cardiac output with modest dilatation of the atria and left ventricle. Hence, N-cadherin is the major cell adhesion molecule responsible for maintaining mechanical and electrical coupling between cardiomyocytes. The severity of the N-cadherin CKO phenotype suggested that reduced levels of N-cadherin might affect gap junction organization thus generating an arrhythmogenic substrate.

In the present study, we examined N-cadherin heterozygous animals with either normal or reduced levels of Cx43 and correlated arrhythmic susceptibility with Cx43 expression. The N-cadherin heterozygous animals exhibited an increased susceptibility to induced arrhythmias accompanied by a reduction in size of the large Cx43-containing plaques. These data provide the first genetic evidence that N-cadherin and Cx43 function together to maintain normal electrical coupling in the heart.

Material and Methods

Generation of Compound Heterozygous Mice

The N-cadherin mutant allele lacking exon 1 was generated as previously described (15). The N-cadherin and Cx43 heterozygous mice were intercrossed and genotypes determined by polymerase chain reaction (PCR). The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Oligonucleotides corresponding to the N-cadherin deleted allele 5'-TGC TGG TAG CAT TCC TAT GG-3' and 5'-GTA TGG CCA AGT AAT GGG GAC-3' generated a unique 450 bp PCR product. The Cx43 mutant allele was genotyped as previously described (17). Mice were analyzed in a mixed 129Sv/C57Bl/6J genetic background.

Western Blot Analysis

Western blot analysis was performed with N-cadherin and Cx43 antibodies as previously described (16). Blotting for total Cx43 was performed with a rabbit polyclonal antibody Cx43 (71-0700, Zymed) and dephosphorylated Cx43 was detected using two different mouse monoclonal antibodies that bind selectively to S364/S365 (13-8300, Zymed; Cx43CT1, generously provided by Paul Lampe, Fred Hutchinson Cancer Research Center, Seattle, WA). For normalization of signals, blotting was also performed with anti-GAPDH (6C5, RDI) monoclonal antibodies, followed by blotting with alkaline phosphatase-conjugated secondary antibody, and chemifluorescent processing (ECF, Amersham BioSciences). Densitometry of samples was performed via use of Image-Quant (Molecular Dynamics) software. Results are expressed at mean $\pm$ SD. Two group comparisons were made with the unpaired student *t*-test. A value of $p < 0.05$ was considered significant.

Quantitative Immunofluorescence Analysis

Hearts were isolated from 6 – 8 month old wild-type, N-cadherin $-/+$, Cx43 $-/+$, N-cadherin/Cx43 $-/+$ mice and fixed in formalin. Indirect immunofluorescence was performed on paraffin-embedded sections of hearts as previously described (18). The sections were incubated with mouse monoclonal antibody N-cadherin (3B9, Zymed) and rabbit polyclonal antibody Cx43 (71-0700, Zymed) overnight at 4 C°, and subsequently with Alexa Fluor 488-conjugated goat anti-mouse and Alexa Fluor 555-conjugated goat anti-rabbit IgG antibodies (Molecular Probes) for 1 hour. The double-stained sections were viewed and photographed with Zeiss LMS510 Meta confocal microscope. Immunofluorescent signal was analyzed by quantitative confocal microscopy using methods validated in previous studies (16,19,20). This method is specifically designed to measure the amount of signal at cell-cell junctions by quantifying the number of pixels concentrated in clusters showing high-intensity fluorescence. Ten test areas were analyzed for Cx43 antibody in each of 4 hearts from each genotype. Each test area was scanned within 21389.06 μm^2 (146.25 $\mu\text{m} \times 146.25 \mu\text{m}$) and digitized into a 1024 $\times$ 1024 matrix (1048576 pixels/test area), 1 pixel size equals 0.02 μm^2 . The amount of immunoreactive signal at cell-cell junctions in each test area was expressed as a percentage of total cell area by quantifying the total numbers of pixels in digitized images exceeding prospectively defined signal intensity thresholds, divided by the total number of pixels occupied by tissue. The number and size (pixels) of individual clusters of high intensity signal within each test area were quantified with NIH Image 1.33u software. To determine how the number and size of gap junctions varied as a function of minimal junction size, we used minimal junction sizes of 3, 5, 7, 9, 20, and 40 or more contiguous pixels and chose 5 and 9 pixels to represent the smaller categories. Results are expressed as mean $\pm$ SD. Statistical significance was assessed by two-way ANOVA (Prism 4). Test of significance between groups were performed using Bonferroni's multiple comparisons tests. A value of $P < 0.05$ was considered significant.

In Vivo Electrophysiology Studies

Protocols for the *in vivo* mouse electrophysiology (EP) study have been previously described in detail (21), and a description of the full stimulation protocol is available at www.upenn.med.edu/mcrc/patel. Briefly, each mouse was anesthetized with pentobarbital (0.033 mg/kg IP) and surface ECGs were obtained using 26-gauge subcutaneous electrodes in each limb and the right precordium. A right internal jugular vein cutdown was performed and an octapolar 1.7-French electrode catheter (CIBer mouse EP; NuMED, Inc.) placed in the right atrium and ventricle under electrogram guidance. Standard pacing protocols were used to assess ventricular conduction, refractoriness and arrhythmia inducibility using up to three extrastimuli and burst pacing. Impulses were delivered at twice diastolic threshold with pulse duration of 1.0 ms. Electrograms were displayed on a multichannel oscilloscope recorder (Bard, Inc., Lowell MA) and stored on optical disk for off-line analysis. Results are expressed at mean $\pm$ SD. For the intracardiac parameters, duration of arrhythmia episodes and arrhythmia cycle length statistical significance was assessed by two-way ANOVA (SPSS 12.0). Test of significance between groups were performed using Bonferroni's multiple comparisons tests. The numbers of arrhythmic episodes were assumed to have a Poisson distribution and the Kolmogorov-Smirnov test was used to assess statistical significance between groups (SPSS 12.0). A value of $P < 0.05$ was considered significant.

Adult Mouse Ventricular Myocyte Isolation and Immunocytochemistry

Adult murine ventricular myocytes were isolated using the method described by Mitra and Morad (22). Briefly, mice were heparinized, anesthetized with pentobarbital 0.033 mg/kg and hearts excised through a sternotomy. Hearts were mounted on a Langendorff apparatus, perfused with Ca²⁺-free Tyrode's solution for 6 minutes at 3.0–3.5 mL/min. and a temperature of 36°–37°C, followed by 12–15 minutes of perfusion with Ca²⁺-free Tyrode's solution containing: collagenase B & collagenase D (Roche Chemical Co.) plus protease (Fraction IV, Sigma Chemical Co.). When the hearts appeared pale and flaccid they were removed from the Langendorff apparatus and the ventricles were dissected away and kept in Ca²⁺-free Tyrode's solution with 1 mg/ml of bovine serum albumin (Fraction XIV, Sigma Chemical Co.). The ventricles were teased into small pieces and then triturated gently with a Pasteur pipette to dissociate individual myocytes.

Cells were then fixed in 4% paraformaldehyde for 20 minutes and subsequently suspended in PBS for immunostaining as described previously (23). Briefly, cells were blocked/permeabilized in PBS containing 0.1% Triton X-100 and 2% fish oil gelatin (Sigma) and incubated in primary antibody overnight at 4 °C. Primary antibodies were described above plus α -catenin (71-1200, Zymed), β -catenin (71-2700, Zymed), plakoglobin (15, BD Bioscience), desmoplakin (AHP320, Serotec) and ZO-1 (33-9100, Zymed). After washes with same buffer, cells were incubated with secondary antibody described above for 1 h at room temperature and mounted using Vectashield (Vector). Images were collected on a Zeiss LMS510 Meta confocal microscope using Carl Zeiss Imaging software. Images were imported into Adobe Photoshop for cropping and linear contrast adjustment. For myocytes stained with ZO-1 and α -catenin, series of Z-scan images were obtained at 0.5 μ m intervals from the bottom to the top surface of the cells, and a projection image was assembled using NIH Image 1.37v software.

Results

We previously demonstrated that cardiac-specific deletion of N-cadherin resulted in downregulation of Cx43-containing gap junctions and sudden arrhythmic death in mice (15, 16). Based on the severity of this phenotype, we speculated that gap junctions might be affected in animals expressing half the normal levels of N-cadherin thus leading to an intermediate phenotype. Furthermore, if our hypothesis is correct, then reducing Cx43 in the N-cadherin

heterozygous background will further compromise electrical coupling leading to a more dramatic phenotype. To test this idea, N-cadherin and Cx43 heterozygous mice were intercrossed to produce animals with reduced levels of both N-cadherin and Cx43. The resulting progeny were generated in the expected Mendelian frequency (Total progeny 324: WT, 84; N-cad $-/+$, 85; Cx43 $-/+$, 86; N-cad/Cx43 $-/+$, 69) and appeared healthy and indistinguishable from their wild-type littermates. Unless otherwise specified, herein heterozygous will refer to all three heterozygous genotypes, i.e. N-cad $-/+$, Cx43 $-/+$, and N-cad/Cx43 $-/+$.

Quantitative Western blot analysis was performed on protein lysates from whole hearts to examine N-cadherin and Cx43 protein levels in heterozygous animals (Fig. 1). Two representative samples ($n=8$) are shown for each genotype. As expected, N-cadherin and Cx43 heterozygous mice showed a 50% reduction in protein levels corresponding to loss of one wild-type allele. Connexin 43 is phosphorylated when it forms an active gap junctional complex at the cell surface. In contrast, it is dephosphorylated during trafficking/endocytosis in the cytoplasm. To directly examine the phosphorylation status of the protein, an antibody was used that recognizes Cx43 only when the large cytoplasmic tail is unphosphorylated at particular serine residues (24). Interestingly, reduction of N-cadherin led to an increase in the dephosphorylated Cx43 consistent with less functional gap junctions in the N-cadherin heterozygous animals. Furthermore, the effect on Cx43 dephosphorylation was exacerbated in the compound heterozygous animals (Fig. 1). Similar results were obtained using an antibody that recognizes hypophosphorylated Cx43 at S364/S365 (25) indicating that multiple serine residues were affected in the heterozygous hearts (Supplemental data).

Based on histologic analysis, the cytoarchitecture of the heterozygous hearts ($n=4$) appeared normal compared to wild-type (data not shown). No increase in fibrosis, as demonstrated by Masson trichrome stain, was observed in the heterozygous hearts compared to wild-type (data not shown). Furthermore, no difference in the heart weight to body weight ratios was observed in heterozygous animals compared to wild-type littermates (WT, 4.27; N-cad $-/+$, 4.26; Cx43, 4.24; N-cad/Cx43 $-/+$, 4.33; $n=8$, $p>0.05$). To assess cardiac function, cardiac-gated magnetic resonance imaging was performed on N-cadherin heterozygous mice. Quantitative analysis of image data demonstrated normal cardiac function compared to wild-type animals (data not shown). Furthermore, transmission electron microscopy of N-cadherin heterozygous hearts did not reveal any apparent abnormalities in the intercalated disc structure or myofibril organization compared to wild-type (Supplemental data). We conclude from these morphological and functional analyses that N-cadherin haploinsufficiency does not cause cardiomyopathy in mice.

The increase in dephosphorylated Cx43 in the heterozygous hearts suggested less efficient gap junction communication between the cardiomyocytes which may contribute to arrhythmogenesis. To assess arrhythmia susceptibility (26), invasive electrophysiological studies were performed on heterozygous and wild-type animals. The heterozygous animals revealed no significant differences in intracardiac electrophysiological parameters (Supplemental data) compared to wild-type mice, however they did exhibit increased vulnerability to inducible ventricular tachycardia (Table I). Specifically, we found that approximately 30–40% of the single heterozygous animals (3 out of 10 N-cadherin, and 4 out of 10 Cx43) had inducible ventricular arrhythmias which increased in the compound heterozygous animals to 60% (6 out of 10 mice, Table I). Representative example of induced ventricular arrhythmia from a N-cad/Cx43 compound heterozygous mouse is shown in Figure 2. Interestingly, the frequency of induced arrhythmias was similar between N-cadherin and Cx43 heterozygous animals. Most importantly, reduction of N-cadherin on the Cx43 heterozygous background led to an increase in arrhythmias compared to the single mutant animals, providing evidence that N-cadherin and Cx43 function together to maintain normal electrical coupling in the heart.

To understand how reduced N-cadherin levels may affect Cx43-containing gap junctions, quantitative immunofluorescence microscopy was performed on heart samples from heterozygous animals. Sections of ventricular myocardium from four animals of each genotype were immunostained with Cx43 antibodies (Fig. 3A–H). For these experiments N-cadherin was used as a marker of the ICD. To determine how the number and size of gap junctions varied as a function of junction size, minimal junction sizes of 5 and 9 or more contiguous pixels were compared (Fig. 3I). When N-cadherin heterozygotes were compared to wild-type mice, we found no significant change in the number of Cx43-containing clusters. However, the average size of the larger (i.e. 9 pixels or more) Cx43-containing gap junctions was reduced (~20%) in the N-cadherin heterozygous animals. In addition, the area occupied by the large plaques was reduced (~11%) in the N-cadherin heterozygotes. As expected, the number and area occupied by Cx43-containing plaques was reduced (~50%) in the Cx43 heterozygotes compared to wild-type animals. Interestingly, the average size of Cx43-positive plaques of 9 pixels or more was similarly reduced in both N-cadherin and Cx43 heterozygotes, providing a possible molecular explanation for the similar frequency of induced arrhythmias in the single mutant animals. Gap junction plaque size was further reduced (~40%) along with total area occupied (~74%) by Cx43 immunoreactivity in the N-cad/Cx43 compound heterozygous animals compared to wild-type littermates which illustrates the intimate relationship between adherens junctions and gap junctions.

To further examine the expression and distribution of Cx43 in the N-cad/Cx43 compound heterozygous animals, immunofluorescence microscopy was performed on isolated adult ventricular myocytes. Representative images were chosen after examining a minimum of ten immunostained cells of each genotype. Similar to our *in situ* results, N-cadherin and Cx43 were reduced at the ICD in the N-cad/Cx43 compound heterozygous cardiomyocytes compared to wild-type (Fig. 4; Supplemental data). The cadherin associated protein, β -catenin, was also reduced at the ICD consistent with N-cadherin being the only classical cadherin expressed in cardiomyocytes (Fig. 5; Supplemental data). In addition, the cytoskeletal linker protein, α E-catenin, was reduced at the ICD in N-cadherin heterozygous cardiomyocytes (Supplemental data). In diseased myocardium, there is often an increase in hypophosphorylated Cx43 along with its redistribution to the lateral borders of the cardiomyocytes (27). The N-cad/Cx43 compound heterozygous cardiomyocytes exhibited a significant increase of hypophosphorylated Cx43 at the ICD and lateral borders of the mutant cardiomyocytes (Fig. 5). In comparison, there was relatively little hypophosphorylated Cx43 in the wild-type cardiomyocytes.

The linkage between the cadherin/catenin complex and gap junctions is thought to be indirectly through an adaptor protein. The cytoskeletal scaffold protein, zona occludens-1 (ZO-1), which binds to the carboxy tail of Cx43 (28) and colocalizes with N-cadherin (29) may mediate this interaction between the two junction complexes. Recent data suggest that ZO-1 regulates Cx43 plaque size (30), therefore we speculated that ZO-1 might be affected in our N-cadherin heterozygous cardiomyocytes where plaque size is significantly reduced (Fig. 3). Indeed, a significant reduction in ZO-1 expression was observed at the ICD in the N-cadherin heterozygous cardiomyocytes compared to wild-type and Cx43 heterozygous cells (Fig. 6).

In addition to adherens junction proteins, desmosomal proteins were lost from the ICD in the cardiac-specific N-cadherin CKO mouse (15) making it difficult to distinguish which adhesive junctions were required for proper gap junction organization. Therefore, we examined the expression of the desmosomal proteins, desmoplakin and plakoglobin, in isolated adult cardiomyocytes. The expression and distribution of desmoplakin and plakoglobin were similar in N-cad/Cx43 compound heterozygous and wild-type cardiomyocytes (Fig. 7) indicating that the effects on Cx43 are likely due to reduction in the cadherin/catenin complex in the N-cadherin heterozygous mice.

Discussion

The ICD contains extremely large Cx43-containing gap junction plaques compared to non-cardiac tissue, which reflects their important function in electrically coupling cardiomyocytes. In the present study, we demonstrate that decrease in the ICD protein, N-cadherin, to half its normal level leads to a reduction in size of the large Cx43-containing plaques in the myocardium of N-cadherin heterozygous mice. The change in gap junction organization could not be attributed to structural remodeling because it occurred in the absence of any functional or histological evidence of cardiomyopathy. The reduction in gap junction plaque size was accompanied by an increased susceptibility to induced arrhythmias in the N-cadherin heterozygous mice. Both phenotypes, reduction in plaque size and susceptibility to arrhythmias, were exacerbated in N-cad/Cx43 compound heterozygotes; this is consistent with the hypothesis that compound heterozygotes have a more labile arrhythmogenic substrate when both N-cadherin and Cx43 are reduced in the myocardium. Interestingly, the frequency of induced arrhythmias was similar between N-cadherin and Cx43 heterozygous mice suggesting that either destabilization or reduction in gap junctions at the plasma membrane is sufficient to generate an arrhythmogenic substrate. The common feature between the N-cadherin and Cx43 heterozygous animals was a similar size reduction (20% of wild-type) in Cx43 plaques with 9 pixels or more, suggesting that large plaques were more important than the total number of plaques which were unaffected by reduction of N-cadherin alone. The further reduction in number and size (40% of wild-type) of the Cx43-containing plaques likely explains the increase in susceptibility to arrhythmias in the N-cad/Cx43 compound heterozygous animals compared to single mutants. Exceptionally large Cx43 plaques (20 and 40 pixels or more) were also affected in the heterozygous mice (Supplemental data). The data were similar to 9 pixels or more (Fig. 3), however a reduction in number of plaques was now observed between N-cadherin heterozygous and wild-type mice. The average size of Cx43 plaques remained similar between N-cadherin and Cx43 heterozygous mice even in this exceptionally large plaque category further supporting the idea that N-cadherin regulates gap junction organization.

Changes in Cx43 phosphorylation regulate Cx43 trafficking, assembly, gating and turnover in the cell (31). A less phosphorylated Cx43 species referred to as the P0 isoform following protein analysis by SDS/PAGE is associated with a reduction in gap junction plaques at the ICD in diseased myocardium. We previously demonstrated an increase in dephosphorylated Cx43 in N-cadherin-deficient hearts, which is associated with reduced Cx43 plaques, reduced epicardial conduction velocity and spontaneous ventricular tachycardia (16). In cultured noncardiac cells, dephosphorylated Cx43 at S364/S365 is associated with trafficking through the Golgi apparatus, hence nonfunctional gap junctions (25). Furthermore, a specific increase in dephosphorylated Cx43 (S364/S365) is observed in protein lysates from ischemic hearts (25). In the present study, an increase in hypophosphorylated Cx43 (S364/S365) was observed in N-cadherin heterozygous hearts, with even higher levels in the N-cad/Cx43 compound heterozygotes, which is consistent with less functional gap junctions and greater susceptibility to induced arrhythmias compared to the single mutants. In future studies, it will be of interest to further examine the cellular distribution and phosphorylation status of Cx43 in cultured neonatal cardiomyocytes derived from N-cadherin heterozygous mice.

N-cadherin is involved in both mechanical and electrical coupling of cardiomyocytes by its ability to organize both myofibrils at adherens junctions and connexins at gap junctions. Cardiac function as determined by magnetic resonance imaging was normal in the N-cadherin heterozygous animals indicating that contractile dynamics were preserved under reduced N-cadherin conditions. In contrast, N-cadherin heterozygous animals were susceptible to induced arrhythmias along with a reduction in Cx43 plaque size. Our results indicate that gap junction organization is particularly sensitive to changes in N-cadherin levels in the heart. The expression and distribution of Cx43 is often perturbed in diseased myocardium, which may be

due, at least in part, to their inherent instability, i.e. 1–2 hour turnover rate for Cx43 in the intact heart (32). The reduced amount of N-cadherin associated with gap junctions in the heterozygous animals may be insufficient to stabilize the larger Cx43 plaques, whereas the total number of plaques was unaffected. There was a modest reduction (14%) in total Cx43 in N-cadherin heterozygous hearts, in comparison Cx43 was reduced 50% in the Cx43 heterozygous hearts where the total number of plaques was reduced. The number of Cx43 plaques assembled appears to be dependent on the absolute amount of Cx43 available.

How does reduction of N-cadherin affect the size of the large Cx43 plaques? The scaffold protein, ZO-1, interacts with the large carboxy terminus of Cx43 via a PDZ domain (28,33), and the cadherin/catenin complex interacts with ZO-1 via α -catenin and the actin cytoskeleton (34) thus forming a molecular linkage between the two protein complexes. ZO-1 is primarily localized with N-cadherin at the ICD, however during gap junction remodeling it is more closely associated with Cx43 (29). In osteoblastic cells, disruption of the Cx43/ZO-1 interaction using a dominant negative ZO-1 leads to a decrease in gap junction function as determined by dye transfer (35). The expression of ZO-1 at the ICD was significantly decreased in the N-cadherin heterozygous ventricular myocytes providing a possible explanation for the reduced size of Cx43 plaques in the mutant cardiomyocytes. The expression of ZO-1 was not affected in Cx43 heterozygous cardiomyocytes consistent with previous studies of ZO-1 in Cx43 CKO hearts (36). Furthermore, recent data correlated reduced ZO-1 with aberrant Cx43 expression in diseased human myocardium (37). The cadherin/catenin complex is important for organization of the actin cytoskeleton and recent evidence has implicated N-cadherin and Rac1 in proper gap junction localization in cultured rat cardiomyocytes (38). Hence, reduced levels of N-cadherin may alter the cytoskeleton leading to decreased stability of gap junctions at the cell surface. Another possibility is that N-cadherin-mediated cell-cell adhesion brings the plasma membranes in close apposition thus creating a localized region of low shear stress, which may be particularly important for assembly and maintenance of large Cx43 plaques (19). Although heart function and structure appear normal in the N-cadherin heterozygous mice, subtle changes in mechanical coupling due to reduce N-cadherin-mediated adhesion could affect localized regions of the sarcolemma creating a less favorable environment for large Cx43 plaques. The effect of reduced N-cadherin on gap junction organization appears to be specific to Cx43 since desmosomal protein expression was not affected in the N-cad/Cx43 compound heterozygous ventricular myocytes.

There is limited information concerning N-cadherin expression in diseased human myocardium, and data from animal models of cardiomyopathy have provided different results depending on the model. Abnormal ICD structure associated with downregulation of N-cadherin and Cx43 was observed in a hereditary hamster model of dilated cardiomyopathy (39,40). In a transgenic mouse model of human desmin-related cardiomyopathy, gap junction remodeling was accompanied by reduced N-cadherin at the ICD (41). In a guinea pig chronic pressure overload model, β -catenin was redistributed from the plasma membrane to the cytoplasm; however, there was no change in N-cadherin (42). In the human disease, Naxos, where the cadherin binding partner plakoglobin is truncated and Cx43 expression is reduced in the heart, N-cadherin expression appears normal in those patients (43). Given these different results, additional work will be required to determine the role of N-cadherin in gap junction dysfunction in cardiomyopathy.

Interestingly, the reduction in number, size, and area occupied by Cx43-containing aggregates in the N-cad/Cx43 compound heterozygous animals is comparable to the cardiac-specific N-cadherin knockout (CKO) phenotype (16). However, the N-cad/Cx43 compound heterozygous animals do not suffer from spontaneous SCD as do the N-cadherin CKO mice, therefore we conclude that a reduced number and size of gap junctions is sufficient to maintain electrical coupling in mice under normal conditions as long as mechanical coupling is maintained at the

ICD. In future studies, it will be informative to determine whether N-cad/Cx43 compound heterozygous animals are more susceptible to SCD in response to cardiomyopathy.

In conclusion, these genetic data demonstrate that decreasing gap junction plaque size was associated with increasing arrhythmogenicity in the absence of cardiomyopathy. Our results suggest that N-cadherin function may be perturbed in diseased myocardium leading to altered gap junction organization thus generating an arrhythmogenic substrate. In the future, screening for human N-cadherin mutations, or other changes affecting its function in cardiovascular disease, may prove useful as a predictor of patients at increased risk of SCD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

N-cadherin and Cx 43 expression in heterozygous mice. A, Quantitative Western blot analysis was performed on heart lysates from wild-type (WT), N-cadherin (N), Cx43 (C), and N-cad/Cx43 compound (N/C) heterozygous adult mice. Blots were probed for N-cadherin, Cx43, and dephosphorylated Cx43 (13-8300). B, Eight independent animals were examined for each genotype and compared with WT. GAPDH signal was used to normalize for loading differences between lanes. The molecular weights for the phosphorylated Cx43 (P) and nonphosphorylated Cx43 (P₀) are 44 – 46 kD and 42 kD, respectively. *, $P < 0.05$.

Figure 2.

Ventricular arrhythmia in N-cad/Cx43 compound heterozygous animal. Shown from top to bottom are surface ECG leads I, II and aVF, along with intracardiac recordings from the right atrium (RA) and right ventricle (RV). Episode of ventricular tachycardia induced by programmed stimulation in a N-cad/Cx43 compound heterozygous animal with the pathognomonic characteristic of rapid ventricular beats and atrial dissociation. Programmed stimulation is delivered as a train of impulses at 120 ms (S1) followed by two extrastimulus (S2) and (S3) at 40ms and 20ms respectively. Note sinus rhythm before and after the conclusion of the arrhythmia, for comparison, where the p-wave (P) represents atrial depolarization followed by ventricular depolarization (QRS).

Figure 3.

Comparison of Cx43 staining patterns in wild-type and mutant animals. Ventricular myocardium from wild-type (WT), N-cadherin (N-cad $-/+$), Cx43 (Cx43 $-/+$), and compound (N-cad/Cx43 $-/+$) heterozygous adult mice was coimmunostained with Cx43 (red, A–D) and N-cadherin (green, E–H merged). Quantitative immunofluorescence microscopy was performed on Cx43-immunostained sections of ventricular myocardium from four independent animals of each genotype (I). Ten test areas from each animal were examined for 5 and 9 or more contiguous pixels of high signal intensity. The number of Cx43-containing clusters or aggregates, the average size of the Cx43-containing clusters, and the area fraction occupied by Cx43-containing clusters in each category is presented. The error bars represent standard deviation of the mean. WT, wild-type; N, N-cadherin $-/+$; C, Cx43 $-/+$; N/C, N-cadherin/Cx43 $-/+$. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. ns, not significant. The P values for all pairwise comparisons are presented in Supplemental data.

Figure 4.

N-cadherin and Cx43 expression in isolated cardiomyocytes. Cardiomyocytes were isolated from adult wild-type (WT) and double mutant (N-cad/Cx43 $-/+$) animals and coimmunostained for N-cadherin (A,C) and Cx43 (B,D).

Figure 5.

Expression of β -catenin and hypophosphorylated Cx43 in isolated cardiomyocytes. Cardiomyocytes were isolated from adult wild-type (WT) and double mutant (N-cad/Cx43 -/+) animals and coimmunostained for β -catenin (A,C) and dephosphorylated Cx43 (13-8300; B,D). Note the overall increase in hypophosphorylated Cx43 at the ICD and lateral side of the cardiomyocyte.

Figure 6.

ZO-1 expression in isolated cardiomyocytes. Cardiomyocytes were isolated from adult wild-type (WT), N-cadherin (N-cad $-/+$), Cx43 (Cx43 $-/+$), and N-cad/Cx43 compound (N-cad/Cx43 $-/+$) heterozygous mice and immunostained for the scaffold protein ZO-1 (A–D). ZO-1 was significantly decreased at the ICD of the cardiomyocytes from N-cadherin (64% versus WT; $P < 0.05$; $n = 8–10$ cardiomyocytes) and N-cad/Cx43 compound (50% versus WT; $P < 0.05$; $n = 8–10$ cardiomyocytes) heterozygous mice. No significant change in ZO-1 was found in Cx43 heterozygous mice compared to wild-type (91% versus WT; $P > 0.05$; $n = 8–10$ cardiomyocytes). Note these pictures were assembled from a series of Z-scan images from the bottom to the top

surface of the cells providing a complete analysis of ZO-1 protein expression in the cardiomyocytes.

Figure 7.

Expression of desmosomal proteins in isolated cardiomyocytes. Cardiomyocytes were isolated from adult wild-type (WT) and double mutant (N-cad/Cx43 $-/+$) animals and immunostained for plakoglobin (A, C) and desmoplakin (B, D).

Table I
Arrhythmic Susceptibility in N-cadherin and Cx43 Heterozygous Mice

	N-cad ^{+/+} ;Cx43 ^{+/+} n=8	N-cad ^{-/+} ;Cx43 ^{+/+} n=10	N-cad ^{+/+} ;Cx43 ^{-/+} n=10	N-cad ^{-/+} ;Cx43 ^{-/+} n=10
Total Episodes VT	1	12 [*]	16 [*]	42, ^{*a}
% of Mice with VT	12.5	30	40	60
VT Duration (s)	0.33	1.24 ± 1.40	1.2 ± 2.13	.805 ± .706
VT CL (ms)	48	42.3 ± 4.5	40.7 ± 8.4	42.6 ± 7.4

VT, ventricular tachycardia; CL, cycle length.

* p<0.05 compared to wild-type

^a p≤0.05 compared to single heterozygotes.

Table II Summary of *p* values from ANOVA analysis of Cx43 expression

Number of Clusters		
<i>p</i> value	5 pixels	9 pixels
WT vs. N	>0.05 ns	>0.05 ns
WT vs. C	<0.001 ***	<0.001 ***
WT vs. N/C	<0.001 ***	<0.001 ***
N vs. C	<0.001 ***	<0.001 ***
N vs. N/C	<0.001 ***	<0.001 ***
C vs. N/C	>0.05 ns	<0.05 *
Average size		
<i>p</i> value	5 pixels	9 pixels
WT vs. N	>0.05 ns	<0.01 **
WT vs. C	<0.05 *	<0.05 *
WT vs. N/C	<0.001 ***	<0.001 ***
N vs. C	>0.05 ns	>0.05 ns
N vs. N/C	<0.01 **	<0.05 *
C vs. N/C	<0.05 *	<0.01 **
Area Fraction		
<i>p</i> value	5 pixels	9 pixels
WT vs. N	>0.05 ns	<0.05 *
WT vs. C	<0.001 ***	<0.001 ***
WT vs. N/C	<0.001 ***	<0.001 ***
N vs. C	<0.001 ***	<0.001 ***
N vs. N/C	<0.001 ***	<0.001 ***
C vs. N/C	<0.001 ***	<0.001 ***