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Original article

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

1. 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

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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.

2. Materials and methods

2.1. 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.

2.2. 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 ± S.D. Two group comparisons were made with the unpaired student *t*-test. A value of $p < 0.05$ was considered significant.

2.3. 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 h. 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 21,389.06 μm^2 (146.25 μm × 146.25 μm) and digitized into a 1024 × 1024 matrix (1,048,576 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