Slow Conduction and Enhanced Anisotropy Increase the Propensity for Ventricular Tachyarrhythmias in Adult Mice With Induced Deletion of Connexin43

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Background—Connexin 43 (Cx43) is a major determinant of conduction in the ventricular working myocardium of mammals. We investigated the effect of decreased Cx43 expression on conduction velocity and arrhythmogenesis using adult mice with inducible deletion of Cx43.

Methods and Results—Cx43 Cre-ER(T)/fl mice, in which 1 coding region of the Cx43 gene was replaced by Cre-ER(T), were mated to Cx43^fl/fl mice, generating Cx43 Cre-ER(T)/fl mice. Application of 4-hydroxytamoxifen (4-OHT) induced Cre-ER(T)-mediated deletion of the floxed Cx43 allele. Epicardial ventricular mapping using a 13 × 19 multiterminal electrode grid (300-μm spacing) was performed on Langendorff-perfused hearts from Cx43^fl/fl plus carrier (n = 10), Cx43^fl/fl plus 4-OHT (n = 10), Cx43 Cre-ER(T)/fl plus carrier (n = 9), and Cx43 Cre-ER(T)/fl plus 4-OHT (n = 10). Cx43 protein amount in group 3 hearts was decreased by ~50% compared with group 1. 4-OHT did not affect cardiac protein amounts in group 2 but decreased Cx43 expression up to 95% in group 4 compared with group 3. Epicardial activation of both left ventricle (LV) and right ventricle (RV) during sinus rhythm was similar in all groups. Conduction velocity (CV) changed only in group 4 animals. For RV (LV), longitudinal CV decreased from 38 (35) to 31.6 (33.6) and transverse CV from 24.4 (16.8) to 10.1 (11.3) cm/s. Dispersion of conduction in RV (LV) was increased by 91% (38%). Programmed stimulation resulted in ventricular arrhythmias in group 4 (7 of 10 mice) but never in groups 1 through 3.

Conclusions—Heterozygous expression of Cx43 did not affect ventricular conduction velocity. Up to 95% decrease of Cx43 protein in 4-OHT–treated Cx43 Cre-ER(T)/fl mice reduced conduction velocity and increased dispersion of conduction and propensity for ventricular arrhythmias. (Circulation. 2004;109:1048-1055.)

Key Words: conduction • tachyarrhythmias • mapping

Several cardiac pathologies, such as myocardial infarction and hypertrophy, increase the propensity for arrhythmias and are associated with changes in ionic currents1 and reduced expression of the cardiac gap junction protein connexin 43 (Cx43).2 Gap junctions are major determinants of conduction velocity (CV) and anisotropy, which both have an important role in the genesis of cardiac arrhythmias. The relationship between Cx43 expression and conduction properties of the cardiac impulse has been studied in genetically engineered mouse models. In heterozygous Cx43-deficient mice (Cx43^+/−), cardiac Cx43 protein is reduced to ~50%–5,4 but the effect of an ~50% reduction in Cx43 protein is still debated. One group reported a reduced CV by 23% to 44% without altering the anisotropic ratio (AR), whereas others could not detect any abnormalities.3 To circumvent perinatal death of Cx43-null mice,6 Gutstein et al7 used α-MHC Cre mice to conditionally inactivate Cx43 in cardiomyocytes. These mice developed normally and succumbed to sudden cardiac death between 1 and 2 months of age. Cx43 protein expression was reduced by 95% and led to a significantly slowed left ventricular longitudinal and transversal CV of 42% and 56%, respectively. The animals developed ventricular arrhythmias, but the underlying mechanism was unclear.7 In this model, Cx43 expression is already decreased during cardiac development, which may induce compensatory mechanisms not analyzed so far.

To exclude compensatory mechanisms, we have used a mouse mutant, in which the coding region of Cx43 can be deleted at any given time point by application of 4-hydroxytamoxifen (4-OHT). Induced, Cre-mediated dele-

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tion of Cx43 led to a decrease of up to \(\approx 95\%\) of cardiac Cx43 protein compared with carrier-treated Cx43\(^{\text{Cre-ER(T)})}\) controls and resulted in QRS prolongation and sudden arrhythmogenic death.\(^{7,4}\) Using a high-resolution electrical mapping setup, we compared Langendorff-perfused hearts of carrier or 4-OHT-treated Cx43\(^{\text{Cre-ER(T)}/\text{fl}}\) and Cx43\(^{\text{fl/fl}}\) animals to determine the effect of reduced myocardial Cx43 protein on conduction velocity, its dispersion, and anisotropic ratio. The mechanism by which tachyarrhythmias occur in the 4-OHT-treated Cx43\(^{\text{Cre-ER(T)}/\text{fl}}\) hearts was studied.

### Methods

#### Animals

Cx43\(^{\text{Cre-ER(T)}/\text{fl}}\) embryonic stem cells, in which 1 coding region of the Cx43 gene was replaced by Cre-ER(T), a fusion construct of the Cre recombinase and a specifically mutated version of the ligand binding domain of the human estrogen receptor,\(^{8}\) were transferred into blastocysts to generate Cx43\(^{\text{Cre-ER(T)/fl}}\) mice. These were mated to previously described Cx43\(^{\text{Cre-ER(T)}/\text{fl}}\) mice\(^{9}\) to generate Cx43\(^{\text{Cre-ER(T)}/\text{fl}}\) mice. For phenotypic analysis, Cx43\(^{\text{Cre-ER(T)}/\text{fl}}\) and Cx43\(^{\text{fl/fl}}\) littermates were used. Adult animals (average age, 13 ± 0.4 weeks; bred in the Institute of Genetics, Bonn, Germany) were injected on 5 consecutive days intraperitoneally with 3 to 4 mg 4-OHT dissolved in plant oil (carrier). The following 4 groups of mice were studied: Cx43\(^{\text{fl/fl}}\) plus carrier (n = 10), Cx43\(^{\text{fl/fl}}\) plus 4-OHT (n = 10), Cx43\(^{\text{Cre-ER(T)/fl}}\) plus carrier (n = 9), and Cx43\(^{\text{Cre-ER(T)/fl}}\) plus 4-OHT (n = 10).

#### Immunoblot and Immunofluorescence Analysis

Hearts were shock frozen after mapping studies and used for individual analysis of Cx43 content. Five-micrometer cryosections of hearts were fixed in ice-cold ethanol, stained with 1:2000 diluted polyclonal Cx43 antibodies,\(^{10}\) and detected with Alexa594 conjugated goat anti-rabbit IgG (1:2000). Nuclear staining was performed with 0.2 \(\mu\)g/mL Hoechst33258 fluorescent dye in PBS.

For immunoblot analysis, whole-heart homogenates (20 \(\mu\)g) were separated by 12.5\% SDS-PAGE. Immunoblots were treated with Cx43 antibodies (1:500)\(^{10}\) followed by peroxidase-conjugated secondary anti-rabbit IgG antibodies (1:20 000). Detection of immunoreactivity was performed with the ECL chemiluminescence kit.

#### Preparation of the Hearts and Recording of Electrograms

Mouse hearts were extracorporated for Langendorff perfusion, and extracellular electrograms were recorded with a 247-point multiterminal electrode (19 \(\times\) 13 grid, 0.3-mm spacing), as described before.\(^{11}\)

Recordings were made in sinus rhythm and during stimulation (1-ms duration, twice stimulation threshold) from the center of the grid at a basic cycle length (BCL) of 100 ms. The effective refractory period (ERP), the coupling interval of the shortest premature stimulus that failed to activate the entire heart, was determined for each site of stimulation separately. Every sixteenth stimulus was followed by 1 premature stimulus. Starting at 90 ms, the coupling interval of the premature stimulus was reduced in steps of 5 ms until ERP. Although arbitrary to some extent, conduction block within the recording area was supposed to occur if activation delay between 2 adjacent recording sites was >5 ms (conduction velocity <0.06 mm/ms).\(^{12,13}\)

If arrhythmias were not present spontaneously, the susceptibility for arrhythmias was tested by programmed stimulation in the following sequence. First, 16 basic and 1 premature stimulus 5 ms longer than locally determined ERP were applied. Second, if 1 premature stimulus failed to induce arrhythmias, 16 BCL plus 3 premature stimuli at ERP + 5 ms were applied. Third, 2-second burst pacing at shortest possible cycle length was applied when premature stimulation with 3 premature stimuli failed to induce arrhythmias. Determination of ERP and susceptibility for arrhythmias was performed first for right ventricle (RV) and subsequently for left ventricle (LV) and was identical for all 4 groups.

#### Data Analysis

Unipolar electrograms were transformed into Laplacian electrograms\(^{14}\) to suppress remote signals. The moment of maximal negative dV/dt in the Laplacian electrograms was selected as local activation time. Activation maps were constructed, and dispersion of conduction was calculated.\(^{15}\) Highest and lowest CV was determined from the paced activation maps. Activation times of at least 4 consecutive electrode terminals along lines perpendicular to intersecting isochronal lines were used to determine CVs.

#### Statistics

Multiple-group comparisons were performed using ANOVA with Bonferroni post hoc analysis for continuous data and \(\chi^2\) for categorical data. Two-group comparisons were performed using unpaired \(t\) tests. Values are given as mean ± SEM. \(P < 0.05\) was considered statistically significant.

### Results

#### Characterization of the Mice

Figure 1A shows immunolabeling for Cx43 in ventricular sections of Cx43\(^{\text{fl/fl}}\) mice, carrier-treated (group 1) or 4-OHT-treated (group 2) animals, and Cx43\(^{\text{Cre-ER(T)/fl}}\) animals either carrier-treated (group 3) or 4-OHT-treated (group 4). Cx43 expression was comparable in groups 1 and 2. Because of exchange of 1 Cx43 coding region by Cre-ER(T), in group 3 animals, Cx43 labeling was less intense compared with groups 1 or 2. In group 4 animals, overall Cx43 labeling was largely reduced, but patchy patterns of residual Cx43 expression were found. No obvious differences were observed in the efficiency of Cx43 deletion between left and right ventricle.

Western blot analyses (Figure 1B) of Cx43 protein in whole-heart homogenates revealed no difference between carrier and 4-OHT-treated Cx43\(^{\text{fl/fl}}\) hearts. Carrier-treated Cx43\(^{\text{Cre-ER(T)/fl}}\) hearts displayed decreased Cx43 protein amounts of \(\approx 50\%\) compared with Cx43\(^{\text{fl/fl}}\) mice. In 4-OHT-treated Cx43\(^{\text{Cre-ER(T)/fl}}\) animals, the deletion of the Cx43 coding region resulted in a decrease of Cx43 content of \(\approx 70\%\) to 95\% compared with carrier-treated Cx43\(^{\text{Cre-ER(T)/fl}}\) mice at the time of the experiment (13 ± 0.6 days after first induction).

#### Ventricular Conduction Properties in Mice With Induced Deletion of Cx43

In sinus rhythm, first activation is predominantly found at apico-lateral sites in both RV (70\%) and LV (82\%). The remaining activation patterns are combinations of basal and lateral sites. There was no significant difference in activation patterns between group 1 through 4 animals in RV or LV (not shown).

Figure 2 shows typical activation maps of all groups studied during BCL (100 ms) and premature stimulation at ERP + 5 ms of both LV and RV. Stimulation at BCL from the center of the grid resulted in anisotropic, ellipsoid activation of the epicardium. In LV, the long axis of the activation pattern was from left-down to top-right, whereas in RV it was top-down, representing epicardial myofiber orientation (shown as blue arrows). Fiber orientation was confirmed by histology (not shown). Activation patterns of 4-OHT–treated Cx43\(^{\text{Cre-ER(T)/fl}}\) (group 4) animals show dense crowding of the
Isochrones (Figure 2, bottom right), indicating impaired conduction, being more pronounced on RV than LV. Only group 4 animals exhibit regions of conduction block during premature stimulation. These zones of block were predominantly found in directions perpendicular to the fiber orientation. Conduction block was observed more frequent in RV (8 of 10 group 4 animals) than in LV (4 of 10 group 4 animals).

Figure 3 shows the longitudinal (CV Long) and transverse (CV Trans) conduction velocities of the 4 groups. On RV, CV Long in group 1, 2, and 3 animals was similar, i.e., 38.0 ± 1.5, 37.1 ± 2.5, and 38.4 ± 1.7 cm/s, respectively, but was significantly reduced (31.6 ± 1.7 cm/s) in group 4 animals. The CV Trans on RV was not different between group 1, 2, and 3 animals (24.4 ± 2.4, 21.5 ± 2.4, and 20.9 ± 1.6 cm/s, respectively) but was significantly reduced in group 4 (10.1 ± 1.1 cm/s). On the LV, however, there was no difference in CV Long between the group 1 through 4 animals (35.0 ± 1.6, 33.4 ± 3.4, 38.5 ± 2.5, and 33.6 ± 3.4 cm/s, respectively). The CV Trans of LV was similar in group 1 through 3 animals (16.8 ± 1.5, 18.0 ± 2.0, and 16.9 ± 0.9 cm/s, respectively) and significantly reduced in group 4 animals (11.3 ± 1.4 cm/s).

The anisotropic ratios (AR = CV Long / CV Trans) for RV and LV are illustrated in Figure 3C. The RV did not show a difference in AR between groups 1, 2, and 3 (1.6 ± 0.2, 2.1 ± 0.6, and 1.9 ± 0.2, respectively). However, AR was significantly increased in group 4 (3.8 ± 0.8). Similarly, the AR of the LV was not different in group 1 through 3 animals (2.2 ± 0.3, 2.0 ± 0.2, and 2.3 ± 0.2, respectively) but was significantly increased in group 4 animals (3.1 ± 0.2).

There was no significant difference between ERP in either RV (61.0 ± 3.3, 56.0 ± 3.1, 66.5 ± 1.8, and 56.0 ± 2.4 ms; P = 0.19) or LV (72.0 ± 5.6, 77.5 ± 6.1, 65.5 ± 3.6, and 61.9 ± 3.9 ms; P = 0.11) for group 1, 2, 3, and 4 animals, respectively.

Figure 4 shows that in the RV, the median, the absolute inhomogeneity, and the inhomogeneity index were similar in groups 1 through 3 but significantly increased in group 4 animals during both BCL and premature stimulation. In the LV, no differences were found during BCL stimulation. Premature stimulation significantly increased the absolute inhomogeneity and inhomogeneity index in group 4 animals but did not affect the median.
Arrhythmias in Cx43-Deficient Mice

Ectopic ventricular beats or sustained ventricular tachycardias (VT) were recorded in group 4 (7 of 10 mice) but never in groups 1 through 3. The frequency and characteristics are presented in the Table.

Figure 5 shows the induction of a sustained tachycardia recorded on RV and LV. Panels A through F (reflecting electrograms A through F) show the activation patterns of the last basic stimulus, the following 3 premature stimuli, and 2 tachycardia complexes. The last basic stimulus results in fast conduction in the fiber direction (A). Although activation propagates perpendicular to the fiber direction toward the base of the heart, albeit at a slower speed, propagation toward the apex is impaired, as illustrated by the crowded isochronal lines. This zone of impaired conduction toward the apex extends after premature stimuli and finally becomes large enough to start reentry. The activation maps suggest that initiation of reentry is already initiated after the second extra stimulus. Electrograms in the area of impaired conduction are hardly fractionated.

On the left side of the same heart, a polymorphic VT ensued with meandering patterns of multiple activation wave fronts. Three of 4 hearts that exhibited sustained tachycardias revealed the same phenomenon, i.e., a stable reentry circuit on RV and multiple wave fronts meandering on LV. One heart showed fibrillatory activation on both RV and LV.

Discussion

The novel findings of this study are, first, that during conditional deletion of Cx43 in adult mice, which avoids compensatory mechanisms during development, a 70% to 95%, but not a 50%, reduction of Cx43 protein amount results in reduced conduction velocity, increased dispersion of conduction, and enhanced arrhythmogeneity. Second, in the setting of a general decrease in gap junctional conductance, arrhythmias can be initiated by premature stimuli. During induction, conduction block preferentially occurs in transverse direction. Third, characteristics of the activation patterns during arrhythmias differ strikingly between RV and LV.

Uncoupling, Slow Conduction, and Anisotropy

Exchange of 1 Cx43 coding region by Cre-ER(T) did not result in any changes in conductive properties. This might indicate that in Cx43fl/fl control mice, g_j has a saturating value, both in longitudinal and transverse direction, which does not become a limiting factor when Cx43 expression is reduced by ~50%. This observation is in line with computer simulations in which the relationship between g_j and CV has been shown...
Uncoupling and Arrhythmogenesis

Ventricular tachycardias were exclusively found in 4-OHT–treated Cx43lox/lox animals. Induced, Cre-mediated deletion of Cx43 resulted in conduction slowing and enhancement of anisotropy. The RV tachycardias were attributable to anisotropic reentry on the RV (Figures 5D through 5F). CV is high in the fiber orientation (top-down) but is severely slowed when it turns perpendicular to the fiber orientation. In this specific example, the VT interval was 85 ms, whereas the RV ERP was 65 ms. Such a large excitable gap supports the anisotropic nature of the reentry rather than of the leading circle type.\(^19\) At the onset of VT, conduction block occurred in the transverse direction. This suggests that global uniform uncoupling results preferentially in transverse conduction block, which is in agreement with other studies in which electrical coupling was reduced by pharmacological interventions.\(^20,21\) In contrast, Koura et al\(^22\) found that transverse conduction block occurred in old canine atria, histologically characterized by a wide separation of myocardial bundles attributable to a large amount of fat cell infiltration. The study of Spach et al\(^23\) however, clearly showed that in pectinate muscle, the nonuniform anisotropic characteristics led to longitudinal conduction block. This discrepancy is unclear, but differences in tissue architecture might play a role. It has been shown that the effect of patchy fibrosis on transverse conduction is much greater than that of diffuse fibrosis, even if the amount of fibrosis is the same.\(^24\) Safety of conduction depends on an interplay between cell-cell coupling (connexins), sodium conductance, and tissue architecture. The balance between these parameters is expected to be different among the various studies, which may explain conflicting reports.
Immunofluorescence and immunoblot analysis did not indicate a direct correlation between residual Cx43 amount and occurrence of arrhythmias in our experiments. It is likely that heterogeneity of Cx43 expression together with decrease of Cx43 protein below the heterozygous level allows for the occurrence of ventricular arrhythmias. Three out of 4 group 4 animals that developed sustained VT exhibited a consistent pattern of anisotropic reentry on RV and fibrillatory conduction on the LV. We can only speculate about the difference between RV and LV activation during arrhythmias. For both the RV and LV, lines of conduction block were functional, because they were absent during basic stimulation. Decrease in connexin expression in concert with inhomogeneity in the expression may have generated these areas of functional conduction block. Wall thickness may explain the difference between RV and LV activation during arrhythmias. Because of the small thickness of the RV wall compared with the LV wall, zones of functional block will become transmural, preferably in RV. The transmural zone of block may set up a reentrant circuit, as shown in Figures 5D through 5F. In LV,

**Arrhythmia Inducibility in Group 4 Animals**

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Minimum Protocol Needed for Arrhythmia</th>
<th>Type of Arrhythmia</th>
<th>Ventricle</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–3</td>
<td>1 extrastimulus/3 extrastimulus/burst stimulation</td>
<td>No arrhythmias</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Spontaneous</td>
<td>Premature beats</td>
<td>RV</td>
</tr>
<tr>
<td>5</td>
<td>Spontaneous</td>
<td>Premature beats</td>
<td>RV</td>
</tr>
<tr>
<td>6</td>
<td>3 extrastimuli</td>
<td>Short-run VT</td>
<td>LV</td>
</tr>
<tr>
<td>7</td>
<td>1 extrastimulus</td>
<td>Sustained VT</td>
<td>RV and LV</td>
</tr>
<tr>
<td>8</td>
<td>1 extrastimulus</td>
<td>Sustained VT</td>
<td>RV and LV</td>
</tr>
<tr>
<td>9</td>
<td>Burst stimulation</td>
<td>Sustained VT</td>
<td>RV and LV</td>
</tr>
<tr>
<td>10</td>
<td>Burst stimulation</td>
<td>Sustained VT</td>
<td>RV and LV</td>
</tr>
</tbody>
</table>

**Figure 4.** Bar plot of the inhomogeneity of conduction for groups 1 through 4 on the RV and LV during BCL (S1–S1=100 ms) and premature stimulation (S1–S2 at ERP +5 ms).
zones of functional block are present as well, but because of the thickness of LV, these zones are often not transmural, allowing activation to proceed via deeper layers, albeit with increased delay. Activation is impaired and irregular, giving rise to fibrillatory conduction. In summary, our results show that a 50% decrease in cardiac Cx43 protein compared with control mice does not affect conduction velocity, anisotropy, and arrhythmogenicity. Additional decrease of Cx43 protein up to 95% slows conduction and enhances anisotropy. In such hearts, arrhythmias are common and based on anisotropic reentry on the RV and fibrillatory conduction on the LV.

**Clinical Relevance**

Reduced expression and redistribution of Cx43 is a common observation during myocardial infarction and hypertrophy, and this remodeling of Cx43 expression is thought to form an anatomic substrate for arrhythmias. In this study, we have shown that a decrease of Cx43 protein below the heterozygous level is needed to affect conduction velocity and arrhythmogenesis. Even at Cx43 expression levels that are barely detectable by immunofluorescence, conduction velocity is only moderately reduced by 15% to 25%. These data indicate that the reduction of Cx43 expression found in heart diseases presumably is not sufficient to affect conduction velocity and arrhythmogenicity. However, heterogeneity in Cx43 expression and reduced expression in concert with increased collagen deposition may be responsible for increased arrhythmogenicity in diseased hearts.

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**References**

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