Rationale: Although connexin changes are important for the ventricular arrhythmic substrate in congestive heart failure (CHF), connexin alterations during CHF-related atrial arrhythmogenic remodeling have received limited attention.

Objective: To analyze connexin changes and their potential contribution to the atrial fibrillation (AF) substrate during the development and reversal of CHF.

Methods and Results: Three groups of dogs were studied: CHF induced by 2-week ventricular tachypacing (240 bpm, n=15); CHF dogs allowed a 4-week nonpaced recovery interval after 2-week tachypacing (n=16); and nonpaced sham controls (n=19). Left ventricular (LV) end-diastolic pressure and atrial refractory periods increased with CHF and normalized on CHF recovery. CHF caused abnormalities in atrial conduction indexes and increased the duration of burst pacing-induced AF (DAF, from 22±7 seconds in control to 1100±171 seconds, P<0.001). CHF did not significantly alter overall atrial connexin (Cx)40 and Cx43 mRNA and protein expression levels, but produced Cx43 dephosphorylation, increased Cx40/Cx43 protein expression ratio and caused Cx43 redistribution toward transverse cell-boundaries. All of the connexin-alterations reversed on CHF recovery, but CHF-induced conduction abnormalities and increased DAF (884±220 seconds, P<0.001 versus control) remained. The atrial fibrous tissue content increased from 3.6±0.7% in control to 14.7±1.5% and 13.3±2.3% in CHF and CHF recovery, respectively (both P<0.01 versus control), with transversely running zones of fibrosis physically separating longitudinally directed muscle bundles. In an ionically based action potential/tissue model, fibrosis was able to account for conduction abnormalities associated with CHF and recovery.

Conclusions: CHF causes atrial connexin changes, but these are not essential for CHF-related conduction disturbances and AF promotion, which are rather related primarily to fibrotic interruption of muscle bundle continuity. (Circ Res. 2009;105:1213-1222.)

Key Words: atrial fibrillation ■ heart failure ■ fibrosis ■ gap junction ■ connexin

Congestive heart failure (CHF) predisposes to atrial fibrillation (AF), although the underlying mechanisms remain incompletely understood. Ventricular tachypacing produces a clinically relevant animal model of CHF. The atria of dogs with ventricular tachypacing-induced CHF are characterized by structural remodeling, conduction abnormalities and the ability to sustain AF. Myocardial electric continuity is assured by gap junctions, cell-to-cell connections that maintain low-resistance intercellular coupling via specialized hemichannel subunit proteins called connexins. Connexin (Cx)43 and Cx40 are the principal atrial gap junctional subunits; abnormalities in their expression and localization are commonly observed in patients and experimental animals with AF. Phosphorylation of Cx43 can regulate channel assembly, degradation, and conductance. In the ventricles, CHF produces hypophosphorylation of Cx43 and redistribution to lateral cell membranes, associated with proarrhythmic conduction slowing. Little is known, however, about connexin changes during CHF-related atrial remodeling and their role in AF maintenance. The present study was designed to assess the changes in atrial connexin expression caused by tachypacing-induced CHF in the dog.

In initial experiments, we noted significant changes in connexin phosphorylation and sought to understand their role in AF. We previously noted that the cessation of ventricular tachypacing, which is followed by the reversal of CHF, dissociates atrial size and function changes from structural...
remodeling and AF sustainability.\(^{11,12}\) Both structural (particularly tissue fibrosis) and connexin remodeling could contribute to CHF-associated AF. We therefore exploited CHF reversal to assess the reversibility of atrial connexin alterations and evaluate their contribution to CHF-related conduction disturbances and AF maintenance.

**Methods**

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Animal Model**

Animal-handling procedures followed National Institutes of Health guidelines. Animals were prepared and studied as described previously.\(^{11,12}\) Forty-nine mongrel dogs (18 to 34 kg) were instrumented with a right ventricular tachycardiaemulator. Dogs were assigned to 3 groups: (1) pacemaker inactive sham controls (CTL group, \(n = 19\)); (2) 2-week ventricular tachycardia at 240 bpm to induce CHF (CHF group, \(n = 15\)); (3) 2-week ventricular tachycardia followed by 4-week recovery (REC group, \(n = 16\)).

Atrial effective refractory period (ERP) was measured with 10 basic (S1) stimuli. ERP was measured at basic cycle length (BCLS) in the left atrial (LA) appendage of all dogs and at 7 additional sites (BCL, 300 ms) in 5 dogs per group. AF was induced by burst pacing and mean AF duration estimated based on 10 inductions. AF \(\geq 30\) minutes was considered sustained and was cardioverted. Five plastic arrays containing a total of 240 bipolar electrodes were sewn to the atrial epicardial surface.\(^3\) Phase-delay and conduction velocity analyses were performed in 5 dogs per group as previously reported.\(^{3,11,12}\) LA tissue samples were taken from these dogs for histological analysis. Other tissue samples were snap-frozen and stored at \(-80^\circ\text{C}\) for biochemistry and confocal microscopy.

**Histology**

Blocks were sectioned along longitudinal and transverse planes. Sections were cut at room temperature and stained with Masson’s trichrome (\(n = 5\) dogs per group). Microscopic images were captured with a Zeiss Axiosplan-2 Imaging microscope. Tiled micrographs were obtained with a 20× objective and mechanical stage using the MosaicX tile feature of Zeiss Axiovision 4.5 software.\(^{13}\) Fibrous tissue content was quantified as percentage surface area, excluding blood vessel-containing regions. Images were analyzed blinded to group assignment.

**Real-Time RT-PCR**

Total RNA was isolated from CTL, CHF, and REC atrial tissues (\(n = 6\) to 10 dogs per group), then DNase-treated, quantified, and quality-controlled as described previously.\(^{14}\) Real-time RT-PCR was performed with 6-carboxy-fluorescein–labeled fluorogenic TaqMan primers, probes, and universal master mix. Fluorescence signals were detected in duplicate, normalized to 18S ribosomal RNA and quantified. The expression of 18S ribosomal RNA was similar among experimental groups (CTL 0.83 ± 0.09; CHF 1.02 ± 0.08; REC 0.77 ± 0.19; \(P = \text{NS}\)).

**ImmunobLOTS**

Membrane proteins were extracted and processed as described previously.\(^{16,18}\) Phosphorylated and dephosphorylated forms of Cx43 (p-Cx43 and np-Cx43, respectively) were detected with a mouse monoclonal antipan-Cx43 antibody (1/1000, Chemicon) based on molecular masses. Serine 368–phosphorylated Cx43 (368p-Cx43) was specifically detected with a rabbit polyclonal antibody (1/1000, Cell Signaling). Cx40 and GAPDH were detected with rabbit polyclonal anti-Cx40 (1/1000, Chemicon) and mouse monoclonal anti-GAPDH (1/10 000, RDI) primary antibodies. Following application of primary antibodies, membranes were incubated with either antimouse (1/10 000, Santa Cruz Biotechnology) or anti-rabbit (1/10 000, Jackson ImmunoLabs) horseradish peroxidase–conjugated secondary antibodies. Signals were detected by chemiluminescence and quantified by video densitometry. Band intensities are expressed relative to GAPDH. GAPDH expression was consistent among groups (CTL, 0.86 ± 0.04; CHF, 0.81 ± 0.04; REC, 0.90 ± 0.02; \(P = \text{NS}\)).

**Immunofluorescence and Confocal Imaging**

Serial 14-μm cryosections prepared from CTL, CHF and REC were fixed as previously described.\(^{14}\) Slides were incubated with mouse anti–pan-Cx43, rabbit anti–phospho368-Cx43, rabbit anti-Cx40, or rabbit anti–pan-cadherin primary antibodies (all 1/200), followed by AF555-conjugated phalidion (1/200, donkey anti-mouse AF488, and goat anti-rabbit AF647 secondary antibodies (both 1/600), with parallel negative-control studies omitting primary antibodies. Slides were imaged in Z-series every 0.25 μm with a Zeiss LSM-510 inverted confocal microscope. Deconvolved Z-series maximum projections were used to create 3D reconstructions. Tissue analysis was performed at equal magnifications over equivalent tissue areas and thickness, excluding vessel-containing regions, blinded to group assignment.

**Connexin Lateralization**

Connexin lateralization was analyzed using a method based on quantification of the angle formed between the local longitudinal cell axis and the main axis of individual connexin clusters. The ratio between transverse and cell end connexin clusters was used to indicate lateralization (for details, see the Online Data Supplement).

**Fibrosis Orientation**

A novel method to quantify fibrosis in the transverse direction relative to fiber orientation was developed based on the probability density function of angles between the fibrosis cluster major axis and the main axis of individual connexin clusters. For details, see the Online Data Supplement.

**Optical Mapping**

Following a left lateral thoracotomy and heparin administration (5000 U. IM), atlaia were excised and perfused via the circumflex artery with Krebs solution (in mmol/L): 120 NaCl, 4 KCl, 1.2 MgSO4, 7H2O, 1.2 KH2PO4, 25 NaHCO3, and 1.2 CaCl2, saturated with 95% O2-5% CO2 at 20 mL/min. Optical recordings were obtained in the LA roof area in the presence of 2,3-butanedione monoxime (15 mmol/L) and di-4-ANEPPS. A charge-coupled device camera (80×80 pixels, RedShirt Imaging) recorded fluorescence at 1 kHz. Optical signals were recorded during 2-Hz electric stimulation. Experiments were performed in CTL and REC hearts to assess the effects of fibrosis on conduction. After optical mapping, the imaged zones were dissected and stored in formaldehyde for longitudinal tissue sectioning, Masson trichrome staining, and image analysis for fibrous tissue mapping. Maximum (ΔF/Δt) was used to define activation time. Phase analysis was performed with a grid of 20×20 points (every 4×4 points of the charge-coupled device matrix).
Mathematical Model
Simulations of electric propagation were initially performed on 2D rectangular tissue section reconstructions to compare with extracellular mapping results obtained in vivo. The fibrous tissue distribution patterns corresponding to control, CHF, and recovery cases were derived from longitudinally oriented histological sections obtained in individual canine LA images with a color-based segmentation of Masson trichrome–stained images. The simulated tissue measured ∼9.5 × 11.5 mm. Propagation was initiated by applying 2-ms square pulses of 180 μA at a cycle length of 300 ms on a 1-mm² surface at the bottom right corner of the tissue. Phase analysis on a square 56-pseudoelectrode lattice was obtained with activation times at pseudoelectrode points separated by ∼1.5 mm to approach the experimental conditions.

Additional simulations were based on fibrous tissue distribution patterns corresponding to control and recovery cases derived from longitudinally oriented histological sections obtained following optical mapping experiments.

The mathematical model was based on the 2D reaction–diffusion equation:

\[
\frac{\partial I}{\partial t} + \frac{1}{r_a} \frac{\partial (a V)}{\partial r} + \frac{\partial (a V)}{\partial y} = C_m \frac{\partial V}{\partial r} + I_{in}
\]

where \( V \) is the transmembrane potential, \( I_{in} \) the total ionic current, \( a \) the cell radius (\( a = 5 \mu m \)), \( r_a \) the tissue resistivity (\( r_a = 75 \text{ Ohm-cm} \)), and \( C_m \) the cell capacitance (\( C_m = 100 \mu F \)).

The total ionic current was given by:

\[
I_{in} = I_{Na} + I_{Cl} + I_{K} + I_{KCa} + I_{Kb} + I_{Ca} + I_{CAC} + I_{CaCl} + I_{Ca2} + I_{Cl} + I_{Ca3} + I_{Cl}
\]

where \( I_{Na}, I_{K}, I_{Cl}, I_{KCa}, I_{Kb}, I_{Ca}, I_{CAC}, I_{CaCl}, I_{Ca2}, I_{Cl} \) indicate Na⁻, inward-rectifier K⁺, transient-outward K⁺, ultrarapid, rapid, and slow delayed-rectifier K⁺, L-type Ca²⁺, Ca²⁺-dependent Cl⁻, Ca²⁺⁻ pump, Na⁺/Ca²⁺ exchange, Na⁺/K⁺-ATPase, and background Na⁺, Ca²⁺, and Cl⁻ currents, respectively. Fibrosis was modeled by replacing active cells of the discretized 2D substrate by holes with no-flux boundary conditions. Simulations were performed with an operator-splitting and finite element method and 12×12-mm² spatial discretization. Numerical integration was obtained by forward-Euler difference with a 5-μs time step. Phase analysis on a square pseudoelectrode lattice was obtained with activation times at pseudoelectrode points separated by ∼1.0 mm to approach the optical mapping experimental conditions.

Data Analysis
Atrial conduction properties were analyzed as previously described. Activation time delays between each electrode site and neighboring sites were normalized to interelectrode distance and the largest values taken to reflect the activation phase relation at that site. Values were binned to create phase-delay histograms. The phase-delay range between the 5% lowest and 5% largest values (P5–95) represents the difference between fastest- and slowest-conducting zones and is increased by regions of slow conduction. The phase-delay range divided by the median value (P5–95/P50) is a conduction heterogeneity index independent of conduction velocity.

Data are presented as means±SEM. Multiple group comparisons were obtained with 1-way ANOVA or 2-way repeated-measures ANOVA as appropriate. When 1-way ANOVAs revealed significant effects, Bonferroni adjusted pairwise comparisons were performed by multiplying probability values by 3. For 2-way repeated-measures ANOVAs, a mixed model using one repeated main factor was applied. In the case of a significant interaction between factors, contrasts based on the global model were used to compare groups (CTRL, CHF, and REC) within the other main factor. Normality of distribution was verified. A 2-tailed probability value of <0.05 was considered statistically significant.

Results

Hemodynamic Indexes
At open-chest study, pulmonary congestion and pericardial effusions were evident in all CHF dogs but no control or REC dogs. Overall group characteristics and hemodynamic data are presented in Online Table I. Ventricular and arterial systolic and arterial diastolic pressures were reduced in CHF dogs, whereas LV end-diastolic, LA, and RA pressures were increased. Hemodynamic indexes among REC dogs were significantly different from CHF dog values and were statistically indistinguishable from control animals.

In Vivo Electrophysiology
Online Figures I and II show electrophysiological properties at open-chest study. CHF increased LA ERP at all BCLs (Online Figure I, A), a change that reversed with recovery. Group was also a significant determinant of regional atrial ERP, and ERP shortening caused by CHF was regionally variable (Online Figure I, B). Regional ERPs in recovery dogs were significantly shorter than CHF dogs and not significantly different from control.

Mean AF duration (Figure 1A) increased in CHF dogs (1100±171 versus 22±7 seconds in CTL) and remained prolonged in REC dogs (884±220 seconds), despite full hemodynamic and ERP recovery. Phase-delay analyses re-
revealed no significant change in the shortest activation delays (P5; Online Figure II, A) or the median phase-delay (P50; Online Figure II, B), reflecting overall conduction speed. The phase-delay range reflecting slow conduction zones (P5–95; Figure 1B), and the conduction heterogeneity index (P5–95/P50; Figure 1C) were both significantly greater in CHF and REC dogs at all BCLs compared to controls, with no difference between CHF and REC groups. Overall conduction velocities per se were not affected by CHF (Online Figure III), consistent with previous observations.

Connexin Remodeling

Connexin Gene Expression

Cx40 and Cx43 mRNA expression data are presented in Figure 2A and 2B, respectively. Neither Cx40 nor Cx43 mRNA expression differed significantly among groups.

Connexin Protein Expression and Phosphorylation

Figure 3 illustrates the results of Western blot analysis of connexin protein expression. Cx43 was detected by an antibody that reacts with both the phosphorylated (p-Cx43, identified with more slowly migrating, higher-molecular-mass bands9,19,20) and nonphosphorylated (np-Cx43, faster-migrating bands) isoforms and also by an antibody specific to Ser368-phosphorylated Cx43 (368p-Cx43). Figure 3A shows representative immunoblots for Cx40, total Cx43, 368p-Cx43, and GAPDH (from top to bottom, respectively), with mean band intensity data shown in Figure 3B through 3G. Total Cx40 (Figure 3B) and Cx43 (Figure 3C, sum of lower and higher-molecular-mass bands) expression were not significantly different among groups. Expression of the higher-molecular-mass band was significantly reduced and the lower-molecular-mass nonphosphorylated form significantly increased in CHF, with full reversal in REC dogs (Figure 3D). Consistent with the changes in different molecular-mass bands, directly detected 368p-Cx43 expression was reduced by ~73% in CHF and returned to control levels in REC dogs (Figure 3E).

Phosphorylation state and connexin subunit stoichiometry may alter gap junctional communication.5 Comparison of upper (p-Cx43) to lower (np-Cx43) band intensities within individual samples allowed for determination of Cx43 phosphorylation state (Figure 3F). The ratio of p-Cx43(np-Cx43 was reduced ~80% by CHF and returned to control values in REC. Although absolute expression values cannot be accurately compared between connexin isoforms detected by different antibodies (because their respective antibodies probe at different, protein-specific epitopes, and the relative affinities of different antibodies differ), relative expression changes can be determined by comparing Cx40 with total Cx43 within each sample. Cx40/total Cx43 protein ratio was increased ~35% by CHF (Figure 3G), likely because of small but consistent concomitant increases in Cx40 and decreases in total Cx43 expression, which were not sufficient to achieve statistical significance over background noise but which emerged as statistically significant when analyzed as a relative expression ratio. CHF-related changes in both phosphorylation state and Cx40/total Cx43 protein ratio were completely reversed in REC dogs.

Connexin Visualization

Immunofluorescence and confocal imaging of control, CHF, and REC atria allowed visualization of Cx40, total Cx43, and 368p-Cx43 expression and distribution (Figure 4). Costaining with the F-actin intracellular marker phalloidin permitted identification of cell borders. Shown in Figure 4A through 4I are representative 3D reconstructions of the end-to-end intercalated disc region between paired adjoining cardiomyocytes (bottom of each image) and front face views of the gap junction complexes connecting them (top of image). A decrease in 368p-Cx43 with CHF that reverses following recovery from CHF is clearly apparent, with no obvious alterations in total connexins. Quantitative analysis of the mean tissue area data confirms the lack of change in Cx40 and T-Cx43, along with significantly reduced 368p-Cx43 expression (Figure 4J through 4L) in CHF that returned to

Figure 2. Means±SEM of Cx40 (A) and Cx43 (B) mRNA expression (top graphs) and corresponding RT-PCR amplification plots (bottom graphs) in LA tissue samples (n=10 CTL, 10 CHF, and 6 REC dogs).
values not significantly different from control with recovery. Additional images of connexin staining in front face maximum projection views of several individual gap junctions are provided in Online Figure IV.

In normal atrial and ventricular myocardium, Cx43 is primarily localized at end-to-end junctions of adjacent cardiomyocytes. Previous studies have reported redistribution of connexins from cell ends to lateral margins in CHF.9,10,19,20

Figure 3. A, Representative immunoblots of Cx40, T-Cx43 (showing both p-Cx43 and np-Cx43 bands), Ser368-phosphorylated Cx43 (368p-Cx43), and GAPDH bands for atrial tissue samples from 3 CTL, CHF, and REC dogs. (Each lane shows results from one dog, with results shown for 3 separate dogs from each group.) B through E, Means±SEM of Cx40 (B), T-Cx43 (C), larger (phosphorylated) and smaller (nonphosphorylated) Cx-43 (D), and Ser368-phosphospecific Cx43 (E) band intensities relative to GAPDH. F and G, Cx43 phosphorylation state and Cx40/Cx43 expression ratio (n=6/observation for B through G). **P<0.01, ***P<0.001 vs CTL; ††P<0.01, †††P<0.001 vs CHF.

Figure 4. A through I, Immunofluorescent confocal images of 3D reconstructions of the end-to-end intercalated disc regions between adjoining cardiomyocytes in longitudinal section (bottom) and a front face view of the gap junction complex connecting them (top), from 1 dog per group (original magnification, ×42). Red indicates phalloidin; blue, Cx40; green, T-Cx43; yellow, Ser368-phosphospecific Cx43. J through L, Means±SEM of Cx40 (J), T-Cx43 (K), and 368p-Cx43 (L) immunofluorescent area (n=6 to 8 dogs per group, 6 representative fields/dog). **P<0.01 vs CTL; †P<0.05 vs CHF for 368p-Cx43.
Figure 5 shows longitudinally oriented sections with detection by antibodies to Cx40 (Figure 5A through 5C) and total Cx43 (Figure 5D through 5F) and actin myofilaments to assess potential connexin redistribution. Laterally oriented connexins were rare in control conditions (Figure 5A and 5D), consistent with a well-recognized prominent atrial anisotropy ratio of ~10:1, reflecting a paucity of transverse connections. Linear staining of Cx43 at the lateral cell margins greatly increased with CHF (Figure 5E), whereas no apparent lateralization is seen for Cx40 (Figure 5B). With recovery from CHF, the predominantly cell end Cx43 distribution pattern seen in control returned (Figure 5F). Quantitative analysis showed no change in Cx40 lateralization under the conditions studied (Figure 5G) but a statistically significant increase in Cx43 lateralization with CHF that reversed with REC (Figure 5H). Online Figure V illustrates Cx43 lateralization with CHF in images coimmunostained for the gap junction marker cadherin.

Tissue Fibrosis

Figure 6 shows representative examples of atrial histopathology in control (Figure 6A), CHF (Figure 6B), and REC (Figure 6C) dogs. Control dogs displayed grossly normal atria with small amounts of interstitial fibrous tissue (Figure 6D), in contrast to the substantial interstitial fibrosis in CHF and REC dogs. To assess the potential role of fibrosis in CHF-related conduction abnormalities, we examined the relationship between fibrous tissue deposition and tissue orientation. If interstitial fibrosis simply proceeded along lateral muscle bundle and cell boundaries, typical of reactive fibrosis, fibrotic changes would not be expected to interfere importantly with longitudinal conduction. If, in contrast, there is reparative fibrosis that replaces zones of dead cardiomyocytes within muscle bundles by collagen, interruption of longitudinal cell–cell communication and important disruptions in conduction might be expected. We closely examined tissue sections containing longitudinally oriented muscle bundles to identify the distribution of fibrosis relative to cardiomyocyte-strands. Both CHF and REC dog samples showed transversely oriented fibrosis interrupting longitudinally running fibers (Figure 6B and 6C, arrows). Quantification of transversely distributed fibrosis confirmed a statistically significant, ~10-fold increase of transversely oriented fibrous tissue in CHF and REC versus CTL (Figure 6E).
Mathematical Modeling and Optical Mapping

Connexin-remodeling results indicate that connexin changes cannot explain conduction alterations in REC dogs, because they completely reversed in the recovered condition. This finding points to tissue fibrosis, which did not change with recovery, as a strong candidate to explain abnormal conduction. We used mathematical simulation to assess whether the speculated role of fibrosis is plausible. The results of conduction simulations are shown in Figure 7. Fibrous tissue is represented by blue dots on the 2D atrial grid. Under control conditions (Figure 7A), conduction was smooth, with smooth lines of wave-front propagation. In contrast, with CHF and REC conditions, conduction became much more heterogeneous, with wave-fronts having less smooth frontal boundaries and less discrete isochrones. The results of phase-delay analysis are provided in Online Table II. Consistent with experimental data, P5–95 and the heterogeneity index (P5–95/P50) were increased relative to control in both CHF and REC conditions. These results suggest that tissue fibrosis is sufficient to account for CHF-related changes in conduction indices.

As a final test of the role of fibrosis in conduction slowing, we performed optical mapping experiments in additional CTL and REC dog coronary artery–perfused atrial preparations, followed by mathematical simulations of electric propagation with fibrosis distributions derived obtained from the same preparations. Examples of atrial activation maps from one preparation of each type are shown in Figure 8A and 8B. Corresponding simulations are shown in Figure 8C and 8D, with fibrous tissue represented by white pixels on the atrial grid. There was generally good agreement between optical mapping results and mathematical model simulations. The results of phase-delay analysis are presented in Online Figure VI. Both P5–95 and the heterogeneity index (P5–95/P50) were significantly increased in REC compared to CTL, in both experimental results and mathematical simulations.

Discussion

We found that CHF resulting from 2 weeks of ventricular tachypacing causes Cx43 hypophosphorylation and lateralization, along with increased Cx40/Cx43 expression ratios. These alterations are accompanied by disturbances in local atrial conduction and a substrate for AF maintenance. We then examined corresponding features after recovery from CHF and noted that whereas connexin abnormalities reverse completely on resolution of CHF, atrial conduction abnormalities and AF-maintaining substrates show no significant improvement. These findings suggest that connexin alterations are not essential for atrial conduction disturbances and AF promotion associated with CHF, rather implicating other factors like atrial fibrosis that show no recovery with CHF reversal. In both CHF and REC dogs, atrial fibrosis interrupts cardiac muscle bundles in their longitudinal orientation, and a mathematical model of the effects of fibrosis on impulse propagation provided results consistent with experimentally measured conduction abnormalities.

Relationship to Previous Studies of Gap Junctional Remodeling in CHF

Several studies have described abnormalities in the expression, distribution, and regulation of ventricular connexins in CHF. Absolute Cx43 expression is generally reduced in CHF ventricles,9,20,23–25 likely related to the activation of the mitogen-activated protein kinase c-Jun N-terminal kinase.26 Recent work suggests an important role for defects in Cx43 phosphorylation in CHF-induced ventricular cardiomyocyte uncoupling,9,10,20 thought to be attributable to increased dephosphorylating activity of protein phosphatase-2A colocalized with Cx43.20 Connexin dephosphorylation plays significant roles in targeting connexins to intercalated disks and in regulating connexin conductance.8,10 One study showed
ventricular Cx40 upregulation in CHF, possibly as a compensation for Cx43 downregulation; however, the functional importance of this alteration is uncertain in view of low level ventricular Cx40 expression.

Clinical and experimental studies of gap junctional remodeling in the atria have produced highly discrepant results. The variable findings may relate to technical issues, differences in models, and species studied in experimental work, as well as population-related factors such as underlying heart disease, duration and type of AF, and concomitant drug therapy in clinical studies. The most consistent findings are hypophosphorylation and increased heterogeneity in connexin-distribution. In the present work, we noted atrial Cx43 dephosphorylation and lateralization in CHF. In most previous studies of subjects with AF, the relative participation of AF versus underlying heart disease was unclear. Our dogs had CHF but not AF, so we were able to analyze the effects of CHF on the AF substrate without contamination from AF-induced remodeling. Like Rucker-Martin et al, who studied atrial remodeling in a postmyocardial infarction rats, we observed clear connexin dephosphorylation. Rucker-Martin et al did not measure conduction indices in their rats but reported complete atrial-cardiomyocyte uncoupling (as indicated by Lucifer-yellow dye transfer) in CHF, which is difficult to reconcile with maintained intraatrial conduction (albeit with increased PR intervals). There is evidence for dissociation between alterations in large-solute transmission and electric communication through connexins.

We found no change in conduction abnormalities with recovery from CHF, despite full return of connexin phosphorylation. This observation suggests that connexin dephosphorylation did not contribute significantly to CHF-related atrial conduction-abnormalities and agrees with previous findings indicating that AF-induced atrial connexin remodeling does not produce detectable conduction changes. Ausma et al observed that AF promotion persists beyond the period required for reversal of connexin expression changes in the goat-AF model, consistent with the dissociation we noted between AF promotion and connexin changes during recovery. Like our findings, a recent study reported that AF associated with severe CHF showed an increased Cx40/Cx43 ratio. The same study found elevated Cx43 expression in AF associated with mild CHF but decreased Cx43 in AF with severe CHF, reiterating the notion that changes may depend on the degree and/or type of underlying pathology.

**Potential Significance**

Despite many studies of connexin changes in animal and human AF paradigms, their role in the arrhythmic substrate remains unclear. One of the most consistent findings in AF patients is increased connexin lateralization. Few studies have examined connexin phosphorylation in AF. Cx43 phosphorylation is an important regulatory mechanism for channel assembly, degradation, and conductance. Our finding of reduced Cx43 phosphorylation associated with increased connexin lateralization agrees with observations at the ventricular level in CHF and at the atrial level in rats with atrial dilation postmyocardial infarction. Our study, the first to our knowledge to evaluate the relationship between atrial connexin phosphorylation changes and functional conduction abnormalities/AF substrates, suggests that connexin changes are not required to produce CHF-related conduction disturbances and AF maintenance. This conclusion is consistent with recent studies in which the gap junction conductance-enhancing peptide rotigaptide failed to improve atrial conduction disturbances or AF maintenance in CHF dogs. The lack of a demonstrable contribution of connexin dephosphorylation/lateralization to conduction abnormalities in our CHF/REC atria contrasts with the evidence for a significant role in CHF-induced ventricular conduction abnormalities. This discrepancy may be related to greater fibrosis at the atrial level, which may obscure the contribution of connexin43 dephosphorylation/lateralization. These findings underscore the need for further studies of the interactions between fibrosis and connexin changes in the control of
conduction, as well as analyses of the functional consequences of connexin changes in various AF paradigms.

There is extensive evidence for a role of atrial fibrosis in the AF substrate.\textsuperscript{1,11,12,38} A recent study demonstrated the importance of posterior LA fibrosis in fibrillation wave dynamics of CHF-related AF.\textsuperscript{39} In the present work, we add to the evolving information regarding the role of atrial fibrosis in AF by providing time course evidence, structural observations and mathematical modeling findings to support the primacy of fibrosis in AF-related conduction disturbances in CHF. Our results lend support to the targeting of fibrosis development for AF prevention, an approach that is attracting considerable interest.\textsuperscript{22,38}

Potential Limitations

CHF can result from many etiologies and can show various forms of pathophysiological evolution. We used a specific animal model that mimics clinical tachycardio-myopathies, but caution is necessary relating these findings to other forms of CHF. In addition, fibrosis likely plays a varying role in different pathological forms of AF and is completely absent in some models.\textsuperscript{3} The connexin changes in the present model do not appear to contribute markedly to the AF substrate; however, connexin abnormalities may play a greater role in other AF-promoting pathologies. Furthermore, any animal model of AF is clearly oversimplified compared to complex clinical pathophysiology.\textsuperscript{40}

We studied Cx43 phosphorylation state by examining the ratio of higher- to lower-molecular-weight bands believed to represent phosphorylated and nonphosphorylated-Cx43 respectively, as well as with a Ser368-phosphorylated Cx43-specific antibody. However, in addition to this important site, there are other potentially significant phosphorylation sites on Cx43,\textsuperscript{41} and we cannot be certain that similar changes would have occurred at other sites.

The quantitative relationship between fibrosis and AF remains to be elucidated, as does the effect of the spatial distribution of fibrosis on atrial conduction and AF-susceptibility. The findings of this study relate to the substrate for AF maintenance and do not bear on other determinants of AF occurrence, such as neurohormonal tone and atrial ectopic activity.

Acknowledgments

We thank Marivé Cossette, Jacynthe Laliberté, Nathalie L’Heureux, and Chantal St-Cyr for technical assistance; Gernot Planck for help with mathematical modeling; and France Thériault for secretarial support.

Sources of Funding

This work was supported by the Canadian Institutes of Health Research (CIHR; award MGP 6957), the Quebec Heart and Stroke Foundation, the Fondation Leducq (European-North American Atrial Fibrillation Research Alliance, ENAFRA; award 07/CVD/03), and the Mathematics of Information Technology and Complex Systems (MITACS) network of centers of excellence. B.B. received a CIHR MD/PhD studentship. P.C. holds a Young Investigator Award from the “Fonds de Recherche en Santé du Québec.” G.M. holds a research fellowship from the Heart and Stroke Foundation of Canada and K.N. held research fellowships from Nihon Kohden/St. Jude Medical and Japan Heart Foundation/The Japanese Society of Electrocardiology.

Disclosures

None.

References


Changes in Connexin Expression and the Atrial Fibrillation Substrate in Congestive Heart Failure

Brett Burstein, Philippe Comtois, Georginia Michael, Kunihiro Nishida, Louis Villeneuve, Yung-Hsin Yeh and Stanley Nattel

_Circ Res._ 2009;105;1213-1222; originally published online October 29, 2009;
doi: 10.1161/CIRCRESAHA.108.183400

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/105/12/1213

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2009/10/29/CIRCRESAHA.108.183400.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org/subscriptions/
On-line Materials and Methods

Animal Preparation and In Vivo Studies

Animal handling procedures were approved by the local Animal Research Ethics Committee following National Institutes of Health guidelines. Animals were prepared and studied as described previously.\textsuperscript{1,2} Fifty mongrel dogs (weight, 18 to 34 kg) were instrumented with a unipolar lead inserted into the right-ventricular apex connected to a ventricular tachypacemaker. Dogs were assigned to 3 groups: 1) pacemaker-inactive sham-controls (CTL group, n=19); 2) 2-week ventricular tachypacing at 240 bpm to induce CHF (CHF group, n=15); 3) 2-week ventricular tachypacing followed by 4-week recovery (REC group, n=16).

On open-chest study days, dogs were anesthetized and ventilated mechanically while body temperature was maintained at 37°C with a heating blanket. A median sternotomy was performed, and bipolar electrodes were hooked into the right-atrial (RA) and left-atrial (LA) appendages for recording and stimulation. Hemodynamic data were obtained with fluid-filled catheters and disposable pressure transducers. Atrial effective refractory period (ERP) was measured with 10 basic (S1) stimuli, and the mean of 3 ERP determinations at each basic cycle length (BCL) was used. ERP was measured at multiple BCLs in the LA appendage of all dogs and at 7 additional sites (RA appendage, RA and LA posterior walls, RA and LA inferior walls, and RA and LA sides of Bachmann’s bundle) with a BCL of 300 ms in 5 dogs from each group. AF was induced by burst pacing (10 Hz, 4 times threshold, 2-ms stimuli, 1 to 10 seconds), and mean AF duration was estimated by the average of 10 inductions. AF $\geq$30 minutes was considered sustained and was cardioverted with synchronized DC shock. A 30-minute rest period was then allowed before experimentation was resumed. After 2 cardioversions in a given dog, no further AF inductions were performed. Five plastic arrays containing multiple bipolar...
atrial electrodes (total of 240) were sewn to the atria to cover the entire atrial epicardial surface as previously described. With the use of electrogram data from these arrays, phase-delay and conduction velocity analyses were performed (Cardiomap© system, Research Center, Sacré-Cœur Hospital and Biomedical Engineering Institute, École Polytechnique and Université de Montréal) to evaluate conduction abnormalities in 5 dogs from each group, as previously reported. LA tissue-samples were taken from these dogs for histological analysis. Tissue samples from the remaining animals in each group were snap-frozen in liquid-N₂ and stored at -80°C for biochemical and confocal microscopy analysis.

**Histology**

From each tissue zone, blocks were sectioned along longitudinal and transverse planes. Sections (6-μm thickness) were cut at room temperature and stained with Masson’s trichrome (n=5 dogs/group). Microscopic images were captured with a Zeiss Axioplan 2 Imaging microscope, equipped with a high-resolution digital camera. Tiled micrographs were obtained with a ×20 objective and mechanical stage using the MosaicX tile feature of the Zeiss Axiovision 4.5 software. Fibrous tissue content was analyzed with Sigmascan 5.0 and quantified as percent surface area, excluding blood vessel-containing regions. Images were analyzed by an investigator blinded to group assignment.

**TaqMan Real-Time RT-PCR**

Total RNA was isolated from CTL, CHF and REC atrial tissues (n=6-10 dogs/group), then DNase-treated, quantified and quality-controlled as described previously. DNA-contamination was excluded by reverse transcription (RT)-negative polymerase chain reaction (PCR). First-
strand cDNA was synthesized from 2-μg total RNA with High Capacity cDNA Archive Kits (Applied Biosystems). Real-time RT-PCR was performed with 6-carboxy-fluorescein (FAM)-labeled fluorogenic Cx43 (RefSeq NM_001002951, Gene Expression Assay Cf02625164_g1) and Cx40 (NM_001017442, Cf02625201_s1) TaqMan primers, probes and universal master mix (Applied Biosystems) with the Stratagene Mx3000P sequence-detection system. Fluorescence signals were detected in duplicate, normalized to 18S-ribosomal RNA (Applied Biosystems) and quantified with MxPro QPCR software (Stratagene). The atrial-tissue expression of the 18S-ribosomal RNA reference gene was not different among experimental groups (CTL 0.83±0.09; CHF 1.02±0.08; REC 0.77±0.19, \(P=\text{NS}\)).

**Western-blot Analysis**

Membrane proteins were extracted and processed as described previously.\(^5,6\) CTL, CHF and REC atrial tissues (n=6 dogs/group) were ground in liquid nitrogen and homogenized on ice with a polytron in 3 milliliters of Tris buffer (10-mmol/L, EDTA 5-mmol/L, pH 7.4) with the following enzyme inhibitors: iodoacetamine 1-mmol/L, AEBSF 0.5-nmol/L, aprotinin 10-μg/mL, leupeptin 10-μg/mL, pepstatin 1-μg/mL, and Na3VO4 1-mmol/L. The homogenates were spun at 800×g for 10 min (4°C) to pellet the nuclei and debris, and then at 10,000×g for 10 min at 4°C, and then the supernatants were ultracentrifuged at 105,000×g for 1 hour (4°C). Membrane-pellets were resuspended in extraction solution supplemented with 2% Triton X-100 and stored at -80°C. Protein was quantified by Bradford assay and separated (100 μg/lane) with 10%-polyacrylamide gel-SDS electrophoresis, then transferred to PVDF membranes. Phosphorylated and dephosphorylated forms of Cx43 (p-Cx43 and np-Cx43, respectively) were detected with a mouse monoclonal anti-pan-Cx43 antibody (1/1000, Chemicon), based on their
respective molecular masses. Serine368-phosphorylated-Cx43 (368p-Cx43) was specifically detected with a rabbit polyclonal anti-phospho-Cx43 antibody (1/1000, Cell Signaling), which recognizes its epitope only when the serine-368 of Cx43 is phosphorylated. Cx40 and GAPDH were detected with rabbit polyclonal anti-Cx40 (1/1000, Chemicon) and mouse monoclonal anti-GAPDH (1/10,000, RDI) primary antibodies. Following the application of primary antibodies, membranes were incubated with either anti-mouse (1/10,000, Santa Cruz) or anti-rabbit (1/10,000, Jackson ImmunoLabs) horseradish peroxidase-conjugated secondary antibodies. Primary-antibody specificity was confirmed both by probing the membrane with the secondary antibody only and by pre-incubating the primary antibody with the control peptide. Signals were detected with Western-Lighting chemiluminescence (PerkinElmer) and quantified by video-densitometry. Band intensities are expressed relative to GAPDH intensity from the same sample; atrial GAPDH protein-expression was consistent among groups (CTL 0.86±0.04; CHF 0.81±0.04; REC 0.90±0.02, P=NS).

**Immunofluorescence and Confocal Imaging**

Serial 14-μm cryosections prepared from snap-frozen CTL, CHF and REC (n=5-8 dogs/group using 6 images/dog) atrial tissues were fixed (4%-paraformaldehyde) as previously described. Slides were incubated with mouse-anti-pan-Cx43, rabbit anti-phospho368-Cx43, rabbit anti-Cx40 or rabbit anti-pan-Cadherin primary antibodies (all 1/200), followed by AF555-conjugated phalloidin (1/200), donkey-anti-mouse AF488 and goat-anti-rabbit AF647 secondary antibodies (both 1/600), with parallel negative-control studies omitting primary antibodies. Slides were imaged in Z-series every 0.25-μm with a Zeiss LSM-510 inverted confocal microscope. To observe AF555-, AF488- and AF647-labeling, 543-nmol/L HeNe, 488-nmol/L Ar and
633-nmol/L HeNe lasers were used respectively. Z-series images were deconvolved with Huygens Professional 3.0 software (Scientific Volume Imaging: MLE algorithm, point-spread function derived from 170-nm fluorescent latex beads). Z-series maximum projections were rendered and quantified with the LSM510 software, and used to create 3-dimensional reconstructions with Volocity Visualization 4.0 Imaging software. Tissue analysis was performed at equal magnifications over equivalent tissue areas and thickness, excluding vessel-containing regions, by an observer blinded to group assignment.

**Analysis of Connexin Lateralization**

Lateralization of connexins was analysed using a method based on the quantification of the angle formed between the local longitudinal cell axis and the main axis of individual connexin clusters. Confocal immunoimages were analyzed starting from the deconvolved images. The 10 middle layers of the z-stack were summed to obtained 2-dimensional images of phalloidin and connexins (Cx40 and Cx43) fluorescence. The following steps were applied to the 2-dimensional images:

1) binary images were created by assuming that the normalized fluorescence intensity greater than 0.51 of the Cx images corresponded to pixels with Cx.
2) Cluster analysis using a Cartesian distance threshold (3 µm) was applied to the binary images to define a set of Cx clusters.
3) Ellipses were fit to individual clusters weighting more than 50 pixels in order to determine the center and axis orientation.

Local orientation of myocytes was estimated from the phalloidin images around the center of the fitted Cx cluster ellipses. Two-dimensional FFT of a 401×401 sub-matrix around the centroid of individual clusters was calculated. The presence of T-tubules increases the high-frequency spatial variation of fluorescence in the phalloidin images along the axis orthogonal to
the t-tubules. The angle of the T-tubules (thus transverse to the cardiac fibers) was estimated
from the image 2-dimensional power spectrum by selecting all pixels with power higher than
12% of the maximum peak. An ellipse was then applied to the resulting set of pixels from which
the orientation of the major axis was kept as the T-tubule orientation.

Cx clusters with eccentricity >4 were kept for analysis since determining the major ellipse
axis was unequivocal. Sets of lateral clusters were determined by setting the angle between the
Cx cluster and T-tubule to be greater than \( \pi/3 \) in the \([0, \pi/2]\) interval. The normalized number of
pixels being lateralized over the total number of Cx pixels was then compared between CTL,
CHF, and REC groups by ANOVA and Tukey-Kramer post-hoc test with a \( P\)-value<0.05
indicating statistical significance.

**Evaluation of Fibrosis Orientation**

A novel method was developed to quantify the amount of fibrosis formed in the transverse
direction compared to cardiac fiber orientation. This method was based on the probability
density function of angles between the fibrosis cluster major axis and the local fiber orientation.

The orientation of fibrosis clusters was evaluated from the binary fibrosis mask obtained by
colorimetric analysis of histological slices. Cluster analysis was done based on 4-neighbors
connected pixels of the binary image (Matlab Image Processing Toolbox, Mathworks Inc.).
Cluster area and orientation were computed and only cluster areas greater than 100 pixels were
assessed. Fiber orientation of the digital images was evaluated based on the grayscale intensity
using the Karlon algorithm\(^7\) with a square mask of size \( s = 6 \) and Gaussian variance \( \sigma^2 = 9 \). The
angles between the fibrosis cluster major axis orientation and the fiber orientation were calculated
and mapped between 0 and \( \pi/2 \). The procedure was done on user-selected image regions where
fiber orientation could be determined (non-transverse fiber regions). The number of angles (i.e.
fibrosis clusters) in the interval $[\pi/2-\epsilon, \pi/2]$ with $\epsilon = \pi/6$, was then compared between groups by
ANNOVA and Tukey-Kramer post-hoc test with a $P$-value<0.05 indicating statistical
significance.

**Optical Mapping**

Optical mapping experiments were specifically performed in separate CTL (n=4) and REC (n=5)
dogs. On study days, dogs were administered morphine (2 mg kg$^{-1}$, s.c.), anesthetized with
$\alpha$-chloralose (120 mg kg$^{-1}$, i.v.) and mechanically ventilated. Following a left lateral thoracotomy
and heparin administration (5000 units, i.m.), hearts were rapidly excised and placed in Tyrodes
solution (composition in mM: 136 NaCl, 5.4 KCl, 1 MgCl$_2$6H$_2$O, 5 HEPES, 0.33 NaH$_2$PO$_4$ H$_2$O,
10 glucose, and 2 CaCl$_2$, titrated to pH 7.35 with NaOH) bubbled with O$_2$. The ventricles were
removed at ~1 cm below the AV ring, and the left atrium isolated from the right. The circumflex
coronary artery was cannulated and fixed in position by sutures around the coronary ostia. Left
atrial preparations were transferred to a tissue chamber maintained at 37ºC and perfused with
Krebs solution (composition in mM: 120 NaCl, 4 KCl, 1.2 MgSO$_4$ 7H$_2$O, 1.2 KH$_2$PO$_4$, 25
NaHCO$_3$, and 1.2 CaCl$_2$, saturated with 95% O$_2$-5% CO$_2$) at constant flow (20 mL/min). To
ensure adequate atrial perfusion, all ventricular branches were tied off.

Optical recordings were made in the left atrial roof area from an epicardial surface area of
~1.5×1.5 cm$^2$ in the presence of the motion uncoupler 2,3-butanedione monoxine (15 mmol/L,
Sigma-Aldrich) and the potentiometric dye, di-4-ANEPPS (200 $\mu$L of 1 mg/mL solution, Sigma-
Aldrich). One CCD camera (80×80 pixels, RedShirt Imaging) recorded fluorescence at 1-kHz to
obtain 4-second acquisitions in the presence of two light sources: light emitting diodes (523 nm
wavelength, NTE Inc.) and a quartz tungsten halogen lamp (Newport Inc.). Bipolar electrodes were positioned around the field of view for pacing and recording. Optical maps were recorded during fixed-frequency electrical stimulation (BCL: 500 ms; amplitude 1.5×threshold).

At the end of the optical-mapping experiments, the imaged zones were dissected and stored in formaldehyde before being prepared for tissue sectioning along the longitudinal surface. Sections (6 μm thickness, ~500 μm from epicardial surface) were cut at room temperature, stained with Masson’s trichrome, digitized (Nikon SuperCoolScan 5000) and fibrous-tissue mapping used for mathematical simulations.

Optical mapping data were analyzed with custom software developed with the use of Matlab (Mathworks Inc.). Average activation maps were calculated by averaging the fluorescence signal segment of each beat segmented with the use of the recorded extracellular electrode signal. A moving mean (10 samples) was applied to increase the signal-to-noise ratio. Maximum |dF/dt| was used to define the activation time at all pixels. Phase-analysis was obtained on the mean activation map with a grid of 20×20 points (every 4×4 points of the CCD matrix).

**Mathematical Model**

Simulations of electrical propagation were initially performed on two-dimensional rectangular tissue-section reconstructions to compare with extracellular mapping results obtained in vivo. The fibrous-tissue distribution patterns corresponding to control, CHF, and recovery cases were derived from longitudinally-oriented histological sections obtained in individual canine LA-images with a color-based segmentation of Masson-Trichrome stained images. Larger tissues needed for phase analysis were obtained by tiling the observed segmented fibrosis four
times in each direction (4×4 layout of the image). The simulated tissue measured ~9.5×11.5 mm.

Sub-sampling of the constructed tissue was obtained to provide a pixel-width of 15 microns.

Propagation was initiated by applying 2-ms square pulses of 180-µA at a cycle length of 300 ms on a 1-mm² surface at the bottom-right corner of the tissue. Activation maps were constructed by measuring the time of maximum dV/dt for all cells of the discretized tissue. Phase-analysis on a square 56-pseudoelectrode lattice was obtained with activation times at pseudoelectrode points separated by ~1.5 mm to approach the experimental conditions, yielding 168 phase values (42 maximum phases) for calculation.

Additional simulations were based on fibrous-tissue distribution patterns corresponding to control and recovery cases derived from longitudinally-oriented histological sections obtained following optical mapping experiments. The exact fibrosis layout was determined by a color-based segmentation of Masson-Trichrome stained images. The mean simulated tissue measured ~1.8 cm×1.8 cm. Sub-sampling of the constructed tissue was obtained to provide a pixel-width of 12 microns.

The mathematical model was based on the 2-dimensional reaction-diffusion equation

\[ \frac{a}{2r_i} \left( \frac{\partial^2 V}{\partial x^2} + \frac{\partial^2 V}{\partial y^2} \right) = C_m \frac{\partial V}{\partial t} + I_{ion} \]

where \( V \) is the transmembrane potential, \( I_{ion} \) the total ionic current, \( a \) the cell radius (\( a = 5 \ \mu m \)), \( r_i \) the tissue resistivity (\( r_i = 75 \ \text{Ohm-cm} \)), and \( C_m \) the cell-capacitance (\( C_m = 100 \ \mu F \)).

The total ionic current was given by

\[ I_{ion} = I_{Na} + I_{K1} + I_{to} + I_{Kur,d} + I_{Kr} + I_{Ks} + I_{Ca} 
+ I_{Cl,Na} + I_{pCa} + I_{NaCa} + I_{NaK} 
+ I_{b,Na} + I_{b,Ca} + I_{b,Cl} \]
with $I_{Na}$, $I_{K1}$, $I_{Ko}$, $I_{Kur,d}$, $I_{Kr}$, $I_{Ca}$, $I_{ClCa}$, $I_{pCa}$, $I_{NaCa}$, $I_{NaK}$, $I_{b,Na}$, $I_{b,Ca}$, $I_{b,CI}$ = Na$^+$, inward-rectifier K$^+$, transient-outward K$^+$, ultrarapid, rapid and slow delayed-rectifier K$^+$, L-type Ca$^{2+}$, Ca$^{2+}$-dependent Cl$^-$, Ca$^{2+}$-pump, Na$^+$.Ca$^{2+}$-exchange, Na$^+$.K$^+$-ATPase and background Na$^+$, Ca$^{2+}$ and Cl$^-$ currents respectively. Fibrosis was modeled by replacing active cells of the discretized 2-dimensional substrate by holes with no-flux boundary conditions. Simulations were performed with an operator-splitting and finite-element method$^9$ with 12×12-$\mu$m$^2$ spatial discretization. Numerical integration was obtained by forward-Euler difference with a 5-$\mu$s time step. Propagation was initiated by applying 2-ms square pulses of 180-$\mu$A at a cycle length of 300 ms on a circular surface of 1.2 mm diameter at the bottom-right corner of the tissue. Activation maps were constructed by measuring the time of maximum $dV/dt$ for all cells of the discretized tissue. Phase-analysis$^3,10$ on a square pseudoelectrode lattice was obtained with activation times at pseudoelectrode points separated by ~1.0 mm to approach the optical mapping experimental conditions, yielding ~200 phase values (60 maximum phases) for calculation.

**Data Analysis**

Atrial conduction properties were analyzed as previously described.$^1-3,10$ For phase-analysis, activation-times during 1:1 pacing were analyzed at each electrode site and related to activation-times at neighboring sites. Activation times were normalized to interelectrode distance and the largest values at each site taken to reflect the activation-phase relation between that site and its neighbors. Values at all sites were binned to create a phase-delay histogram. The phase-delay range between the 5%-lowest and 5%-largest values (P5-95) represents the difference between fastest- and slowest-conducting zones and is increased when there are regions of slow
conduction. The phase-delay range divided by the median value of the phase-delay histogram (P5-95/P50) is a conduction heterogeneity index independent of conduction velocity.

Data are presented as mean±SEM. Multiple-group comparisons were obtained with 1-way ANOVA or two-way repeated-measures ANOVA as appropriate. When 1-way ANOVAs revealed significant effects, Bonferroni-adjusted pairwise comparisons were performed by multiplying probability values by 3. For two-way repeated-measures ANOVAs, a mixed model using one repeated main factor was applied. In the case of a significant interaction between factors, contrasts based on the global model were used to compare groups (CTL, CHF and REC) within the other main factor [BCL (150, 200, 250, 300 and 360) or region (RAA, RAPW, RAIW, RABB, LABB, LAPW, LAIW and LABB)]. Before all analyses, the basic assumption of normality of distribution was verified. A two-tailed $P$-value<0.05 was considered statistically significant. Analyses were performed with SAS 9.1.
References


On-line Figure I. Electrophysiological properties at open-chest study. Mean ± SEM LA-appendage ERP at various BCLs (A, n=15 CTL; 15 CHF; and 11 REC; *P<0.05, **P<0.01, ***P<0.001 vs CTL and REC) and in various regions at 300-ms BCL (B, n=5/group; *P<0.05, **P<0.01 for inter-group comparison indicated). RAA, RAPW, RAIW, RABB, LAA, LAPW, LAIW, LABB = right-atrial and left-atrial appendage, posterior wall, inferior wall, Bachmann’s bundle.
A. 5% Smallest Phase-Delays

B. Median Phase-Delay

C. 5% Largest Phase-Delays

**P < 0.01 for inter-group comparison shown.**
Online Figure III. Conduction velocity (mean±SEM) as a function of BCL (A) and atrial region (B). No significant differences were seen (ANOVA).
Online Figure IV. Immunofluorescent confocal images for gap junctions seen in front-face maximum projection views, stained with either Cx40 (A, blue), T-Cx43 (B, green) or 368p-Cx43 (C, yellow).

Original magnification ×63, bar=5 µm.
On-line Figure V. Immunofluorescent confocal images stained for actin (red), Cx43 (green) and cadherin (yellow).

Shown is one longitudinal section each for CTL (left), CHF (middle) and REC (right), with images simultaneously illustrating staining for: actin and Cx43 (top), actin and cadherin (middle), and all 3 (bottom).

Horizontal bar=20 μm. Arrows indicate examples of lateralized staining.
Online Figure VI. Indexes of conduction abnormalities in vitro.

Mean±SEM absolute heterogeneity (P5-95) in optical mapping (A) and simulations on two-dimensional tissue sections (B). Heterogeneity index (P5-95/P50 C, D); n=4 for CTL, n=5 for REC. *P<0.05, **P<0.01 vs CTL.
**On-line Table I.** Hemodynamic Indexes (mm Hg) at Open-Chest Study

<table>
<thead>
<tr>
<th></th>
<th>CTL</th>
<th>CHF</th>
<th>REC</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>15</td>
<td>15</td>
<td>11</td>
</tr>
<tr>
<td>Systolic BP</td>
<td>130±4</td>
<td>106±5**</td>
<td>137±9††</td>
</tr>
<tr>
<td>Diastolic BP</td>
<td>79±4</td>
<td>64±3**</td>
<td>70±4</td>
</tr>
<tr>
<td>LVSP</td>
<td>125±6</td>
<td>102±5*</td>
<td>128±8†</td>
</tr>
<tr>
<td>LVEDP</td>
<td>3.7±0.7</td>
<td>14.5±1.0***</td>
<td>5.1±1.0†††</td>
</tr>
<tr>
<td>LAP</td>
<td>3.7±0.5</td>
<td>13.1±1.4***</td>
<td>4.8±0.6†††</td>
</tr>
<tr>
<td>RAP</td>
<td>2.8±0.4</td>
<td>8.3±0.5***</td>
<td>3.5±0.5†††</td>
</tr>
</tbody>
</table>

Arterial pressure are means during the experiments. Intracavitary pressures (LVSP, LVEDP, LAP, RAP) were measured at the conclusion of experiments following electrophysiological study.  
Abbreviations: BP=arterial blood pressure; LVSP, LVEDP=left-ventricular systolic, end-diastolic pressure; LAP, RAP=left-atrial, right-atrial mean-pressure. *P<0.05, **P<0.01, ***P<0.001 vs CTL; †P<0.05, ††P<0.01, †††P<0.001 vs CHF.
**On-line Table II.** Comparison of phase-analysis results between sham-control (CTL), CHF, and REC simulations

<table>
<thead>
<tr>
<th></th>
<th>$P_{50}$</th>
<th>$P_{5-95}$</th>
<th>Index</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CTL (sham)</strong></td>
<td>1.009 ms/mm</td>
<td>0.704 ms/mm</td>
<td>0.698</td>
</tr>
<tr>
<td><strong>CHF</strong></td>
<td>1.1912 ms/mm</td>
<td>1.1507 ms/mm</td>
<td>0.966</td>
</tr>
<tr>
<td><strong>REC</strong></td>
<td>1.182 ms/mm</td>
<td>1.0481 ms/mm</td>
<td>0.901</td>
</tr>
<tr>
<td>(CHF-CTL)/CTL (%)</td>
<td>18.1%</td>
<td>63.5%</td>
<td>38.4%</td>
</tr>
<tr>
<td>(REC-CTL)/CTL (%)</td>
<td>17.2%</td>
<td>48.9%</td>
<td>29.1%</td>
</tr>
</tbody>
</table>