Atrial Remodeling and the Substrate for Atrial Fibrillation in Rat Hearts With Elevated Afterload

Shang-Jin Kim, DVM, PhD; Stéphanie C.M. Choisy, PhD; Palash Barman, MRCP(Lon); Haifei Zhang, PhD; Jules C. Hancox, PhD, FSB; Sandra A. Jones, PhD; Andrew F. James, DPhil

Background—Although arterial hypertension and left ventricular hypertrophy are considered good epidemiological indicators of the risk of atrial fibrillation (AF) in patients, the link between elevated afterload and AF remains unclear. We investigated atrial remodeling and the substrate for arrhythmia in a surgical model of elevated afterload in rats.

Methods and Results—Male Wistar rats (aged 3–4 weeks) were anesthetized and subjected to either partial stenosis of the ascending aorta (AoB) or sham operation (Sham). Experiments were performed on excised hearts 8, 14, and 20 weeks after surgery. Unipolar electrograms were recorded from the left atrial epicardial surface of perfused hearts using a 5×5 electrode array. Cryosections of left atrial tissue were retained for histological and immunocytochemical analyses. Compared to Sham, AoB hearts showed marked left atrial hypertrophy and fibrosis at 14 and 20 weeks postsurgery. The incidence and duration of pacing-induced AF was increased in hearts from AoB rats at 20 weeks postsurgery. The substrate for arrhythmia was associated with reduced vectorial conduction velocity and greater inhomogeneity in conduction but without changes in effective refractory period. Left atrial expression of the gap junction protein, connexin43, was markedly reduced in AoB compared with Sham hearts.

Conclusions—Using a small-animal model, we demonstrate that elevated afterload in the absence of systemic hypertension results in increased inducibility of AF and left atrial remodeling involving fibrosis, altered atrial connexin43 expression, and marked conduction abnormalities. (Circ Arrhythm Electrophysiol. 2011;4:761-769.)

Key Words: arrhythmia • conduction • connexin43 • fibrosis • gap junctions

Atrial fibrillation (AF) is the most common arrhythmia and is associated with significant mortality, principally through heart failure and stroke.1,2 There are many possible causes of AF, and the risk of AF increases with age; however, the majority (>70%) of patients have some form of structural heart disease.2 There is a close association between the risk of AF and the existence of an elevated afterload. Systemic hypertension and left ventricular hypertrophy (LVH) are considered good epidemiological indicators of AF risk.1–3 Moreover, aortic stenosis is associated with chronic AF.4,5 However, the mechanisms underlying the relation between elevated afterload and the development of AF remain unclear.

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It has been suggested that structural changes to the left atrium (LA) as a result of an underlying pathology, termed atrial remodeling, predispose the heart to AF.2,6 Dilatation and enlargement of the LA is also a good indicator of AF risk,3,7 and it has been proposed that elevated afterload has hemodynamic consequences on the LA, resulting in atrial remodeling and increased risk of AF.5,8,9 Canine models of risk factors for AF associated with hemodynamic overload of the LA, such as congestive heart failure and mitral valve regurgitation, show an increased susceptibility to AF related to interstitial fibrosis and conduction abnormalities.10–12 Long-term systemic hypertension in animal models also have recently been shown to be associated with increased susceptibility to AF,13,14 suggesting that elevated afterload in the absence of heart failure may predispose the heart to AF.

However, it is striking that to date, there is no information about atrial electric remodeling in an animal model of elevated afterload itself. A model of aortic stenosis involving banding of the ascending aorta of weanling male Wistar rats produces LVH without systemic hypertension, in the absence of changes in tissue or plasma norepinephrine levels, and without activation of the systemic renin-angiotensin system.15,16 Accordingly, we have examined the inducibility of and electric substrate for AF of excised perfused hearts from rats with banding of the ascending aorta (AoB) compared with sham-operated controls (Sham).
Methods

Animal Model With Elevated Afterload

All procedures were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986. A gradual increase in LV afterload was achieved by partial stenosis of the ascending aorta in weanling rats using methods derived from Schunkert and colleagues. Briefly, 145 male Wistar rats (B&K Universal Ltd) aged 3 to 4 weeks and body weight of 100 to 120 g were subjected to general anesthesia (80 mg/kg ketamine and 8 mg/kg xylazine), intubated, and ventilated, and a right-side thoracotomy was performed. In 82 of these (AoB), a silk ligature (3–0) was tied around the ascending aorta to the outer diameter of a blunt 20-gauge needle (0.91 mm). The remaining 63 time-matched Sham controls were subjected to the same procedure, except that a suture was not tied around the aorta. Systolic blood pressure and heart rate were measured in conscious animals by tail-cuff plethysmography 1 week before euthanasia (Harvard Apparatus Ltd). Experiments were conducted 8, 14, and 20 weeks postsurgery. Two Sham and 9 AoB rats died during surgery, and a further 3 Sham and 5 AoB rats died before experiments. On the day of experimentation at the end of 20 weeks, 5 AoB rats showed evidence of heart failure (eg, pulmonary enlargement) and were excluded from the study.

Electrophysiological Recordings From Whole Hearts

Hearts were excised under general anesthesia (80–100 mg/kg IP sodium pentobarbital), mounted on a whole-heart perfusion apparatus, and perfused retrogradely through the aorta with a Krebs Henseleit solution (in mmol/L, 118.5 NaCl, 25.0 NaHCO3, 3.0 KCl, 1.2 MgSO4·7H2O, 1.2 KH2PO4, 2.5 CaCl2, 11.1 D-glucose) at 37°C and gassed with 95% O2/5% CO2, as described previously. LA electrograms.17,18 The electrode array was pressed against the epicardial surface of the anterior aspect of the LA free wall. The interelectrode distance was 334 μm; therefore, the recording area was 1.336×1.336 mm. All electrograms were individually amplified and were acquired to a PC hard disk through a Power1401 interface using Spike2 software (Cambridge Electronic Design). Bipolar stimulation was applied through electrodes adjacent to the recording array, and stimulus protocols were generated using an AMPI Master-8 programmable stimulator with Iso-Flex stimulus isolators (Intracel Ltd). Atrial effective refractory period (AERP) was measured using an S1-S2 stimulus protocol. The activation time at each electrode was taken as the time at the point of maximum negative deflection and were measured relative to the earliest fiducial point of activation. Color-coded activation maps with isochrones of 300 μs were generated using OriginPro 8 (OriginLab Corp). The maximal vectorial conduction velocity (CV) across the array was calculated from the electrogram most distal to the earliest fiducial point with the shortest activation time (Figure 1). The phase differences in activation times at each electrode, the absolute inhomogeneity (the range of phase differences from the 5th–95th percentiles), and the index of inhomogeneity (the absolute inhomogeneity divided by the median of the phase differences) were calculated according to the method of Lammers et al. To examine the inducibility of AF, each heart was subjected to 3 consecutive bursts of rapid pacing, each of 5 s duration at cycle lengths of 2, 5, and 10 ms, respectively. The duration of the longest paroxysm of AF induced in each heart, whether by burst pacing or by the S1-S2 protocol, was recorded.

Histology

Cryosections (10 μm thickness) of LA tissue were prepared as described previously. The tissue blocks were oriented for sectioning such that sections were obtained in the frontal plane. Because of the small size and the curvature of the tissue, it was not possible to obtain sections in which all the muscle fibers were oriented in the same plane. For the histological analysis of fibrosis, fixed sections were stained with Masson trichrome. Digitized images were obtained, and the blue pixel content was measured relative to the total tissue area using Adobe Photoshop CS2. Frozen sections were labeled with antibodies using protocols previously described. A total connexin43 (Cx43) antibody (MAB 3068; Chemicon International Inc) and a phospho-Cx43 antibody (3511S; Cell Signaling Technology) were used. Alexa Fluor 488-conjugated antimouse IgG1 secondary (A21121; Invitrogen) was applied at 1 μg/mL. Confocal images were collected using an LSM-510 laser scanning microscope (Carl Zeiss Ltd). Images of equal area were selected at random from Sham (n=5) and AoB (n=5) tissue sections, and Cx43-specific fluorescent staining was quantified using Adobe Photoshop CS2.

Figure 1. Measurement of conduction velocity. A, Representative atrial electrograms. The stimulus artifact is much larger than the scale shown and is therefore not visible. Arrows indicate point of maximal negative deflection of the atrial electrogram. B, On the bottom is a color-coded activation map. Numbers on horizontal and vertical axes indicate index number of electrode. Arrow represents the calculated vector of conduction velocity. Matrices of activation times and distances from earliest fiducial point to electrodes are shown on the top. Numbers within boxes indicate the earliest fiducial point and the point of measurement.
Statistical Analysis

Data are presented as mean±SEM. All data were subjected to D’Agostino-Pearson omnibus K2 normality test. Student t test, 1-way ANOVA with Bonferroni or Kruskal-Wallis with Dunn multiple comparisons post hoc tests, and 2-way ANOVA with repeated measures and Bonferroni post hoc test were performed, as appropriate, using Prism 5.03 (GraphPad Software Inc). The statistical test applied is specified in the figure legends. Differences in the incidence of arrhythmia between the Sham and AoB groups were compared using Fisher exact test. Analysis of correlation was conducted using Spearman rank correlation coefficient. P<0.05 was considered statistically significant.

Results

Aortic Stenosis Produces Atrial Hypertrophy in Rats

AoB resulted in reduced tail-artery systolic pressures (Figure 2Ai) without elevation of heart rate (Figure 2Aii) compared with Sham at 8, 14, and 20 weeks postsurgery in conscious animals, confirming the absence of systemic hypertension in this model.15 Consistent with previous reports of LVH in rats because of aortic banding,15,21 hearts from AoB rats showed marked LVH with an increased LV weight (LVW)/body weight ratio compared with Sham controls (Figure 2Bi). The LVW/body weight ratio was highest in AoB rats at 8 weeks postsurgery. It was reduced in the 20-week AoB compared with the 8- and 14-week AoB groups because of an increase in BW relative to LVW rather than to a reduction in LVW per se (data not shown). AoB-induced LA hypertrophy, which in contrast to the LVH developed with relatively long-standing elevated afterload, showed no significant difference in LA weight/heart weight ratio (LAW/HW) between the AoB and the Sham hearts at 8 weeks, whereas LA hypertrophy was significant at 14 and 20 weeks postsurgery (Figure 2Bii). Measurements of LA width from the anterior to posterior fold at the widest point (Sham, 5.4±0.2 mm; AoB, 6.8±0.1 mm; P<0.0001) and of LA length from left to right at the longest point (Sham, 6.8±0.2 mm; AoB, 8.8±0.3 mm; P<0.001) in 6 Sham and 9 AoB hearts indicated significant LA enlargement at 20 weeks postsurgery. The absence of a significant difference between AoB and Sham in systolic aortic pressures in excised-perfused hearts at 14 and 20 weeks postsurgery demonstrates that aortic banding did not result in LV failure in these hearts (Figure 2C).

Inducibility of AF

The inducibility of AF in excised-perfused hearts from each group was examined using burst and S1-S2 pacing protocols.
A more homogeneous appearance with a single broad wave-reversion to sinus rhythm, however, excitation resumed to the center of the array (Figure 4A and 4B). After spontaneous evidences wherein the earliest point of activation appeared close to the center of the array could be observed, and apparently focal-like activity was seen (Figure 4). Before induction of the arrhythmia, excitation proceeded as a broad single wavefront beneath the electrode surface of the LA before, during, and after the paroxysms of AF (Figure 3). Atrial arrhythmia was not induced in any of the hearts from Sham at 8 weeks postsurgery (Figure 3A). In contrast, brief paroxysms of AF, which spontaneously reverted to sinus rhythm after a few seconds, could be induced in hearts from each of the other groups (Figure 3A). The incidence of AF in AoB hearts at 20 weeks postsurgery was significantly greater than in time-matched Sham controls or AoB rats earlier (8 and 14 weeks) postsurgery (Figure 3A). Thus, long-term elevation of afterload was associated with an increased inducibility of AF. However, when AF could be initiated, there was no statistically significant difference between the groups in its duration (Figure 3B).

Conduction maps were constructed from the epicardial surface of the LA before, during, and after the paroxysms of AF (Figure 4). Before induction of the arrhythmia, excitation proceeded as a broad single wavefront beneath the electrode array in both Sham (Figure 4A) and AoB (Figure 4B) hearts. In contrast, during the arrhythmia, the normal pattern of conduction was considerably disrupted; multiple wavefronts could be observed, and apparently focal-like activity was evident wherein the earliest point of activation appeared close to the center of the array (Figure 4A and 4B). After spontaneous reversion to sinus rhythm, however, excitation resumed a more homogeneous appearance with a single broad wavefront indistinguishable from that which was evident before the arrhythmia (Figure 4).

The Proarrhythmic Substrate
AERP, measured from the LA using an S1-S2 protocol, showed a clear dependence on cycle length in hearts from each group (Figure 5A). Note that as a consequence of the cycle length-dependent shortening of AERP, not all hearts were able to follow the S1 stimuli at a cycle length of 75 ms. Consequently, data are missing at this cycle length (indicated by bracketing of the data in Figure 5). For this reason, the mean data at a cycle length of 75 ms were not included in the analysis by 2-way ANOVA with repeated measures. In any case, there was no difference between the time-matched groups in AERP at any cycle length. The LA vectorial CV also showed strong cycle length dependence (Figure 5B). In contrast to AERP, there were significant differences in CV between the groups, with conduction being significantly slower in 20-week AoB hearts than in the corresponding time-matched Sham hearts. As a consequence of the reduction in CV, the wavelength of excitation also was shortened in 20-week AoB hearts compared with time-matched Sham hearts (Figure 5C). Conduction maps constructed during normal excitation in sinus rhythm provided further evidence of conduction abnormalities in the AoB hearts (Figure 6A). The index of inhomogeneity, calculated from the phase differences during normal sinus excitation, was significantly greater in the AoB hearts at 20 weeks postsurgery than in age-matched controls and with AoB hearts at 8 weeks postsurgery (Figure 6B). Moreover, the index of inhomogeneity correlated significantly with the duration of arrhythmia in the 17 hearts in which AF was induced (r=0.674, P=0.003) (Figure 6C). These observations further support the notion of a proarrhythmic substrate that developed with long-standing elevated afterload.

Fibrosis and Gap Junction Remodeling
Interstitial fibrosis and changes in lateral coupling through gap junctions have been suggested to contribute to conduction abnormalities and a substrate for atrial arrhythmia in structural heart disease. Concordant with this, the degree of fibrosis evident in Masson-stained sections of LA tissue (Figure 7A) was significantly greater in tissue from AoB rats at 20 weeks postsurgery than either time-matched Sham controls or AoB rats earlier (8 and 14 weeks) postsurgery (Figure 7B). Cx43 represents the predominant myocardial gap junction subunit, and changes in the expression and phosphorylation of Cx43 protein in animal models of heart failure have been associated with proarrhythmic remodeling. Therefore, we investigated whether changes in Cx43 protein expression were evident in AoB hearts after long-standing afterload elevation by immunolabeling of Cx43 protein in frozen sections of LA from Sham controls and AoB hearts at 20 weeks postsurgery (Figure 8). Cell membranes were stained using wheat germ agglutinin. In Sham atria, Cx43 was localized in dense clusters at the ends of the cells, and there was no difference in the pattern of staining obtained using either the total Cx43 or the phospho-Cx43-specific antibody (Figure 8A and 8C); these observations were con-
consistent with the majority of Cx43 protein being phosphorylated and localized to the intercalated disks in normal myocardium. However, the density of labeling with either total Cx43 or phospho-Cx43-specific antibodies was significantly reduced in sections from time-matched AoB rats compared with Sham controls (cf, Figure 8B, 8D, and 8E). Collectively, these data constitute evidence for increased fibrosis and remodeling of gap junction proteins in long-term elevated afterload.

**Discussion**

The findings of the present study provide novel evidence of a key role for chronically elevated afterload in the predisposition to AF. The remodeling of the LA (eg, LA hypertrophy,
conduction abnormalities, fibrosis) and the increased susceptibility to AF in hearts from rats with aortic stenosis occurred in the absence of hypertension or heart failure. Thus, long-standing elevation of afterload in the absence of heart failure causes remodeling of the LA and the development of a substrate for arrhythmia.

LVH, Atrial Enlargement, and AF
Although the existence of a hypertrophied left ventricle is considered a good epidemiological indicator of AF risk in patients\(^1-3\) and a marked degree of LVH was evident in the AoB hearts in the present study, the LVW/body weight ratio was not significantly correlated with the duration of AF \(r=0.218\), indicating that the degree of LVH is not directly related to the predisposition of the atrium to AF in this model. The atrial remodeling was not evident at 8 weeks postsurgery, when the greatest degree of LVH was observed, but required long-term elevation of afterload so that the greatest remodeling was evident at 20 weeks. Thus, the data are consistent

Figure 5. Cycle length dependence of atrial electrophysiological parameters in AoB and Sham hearts. A, AERP. B, Conduction velocity (CV). C, Wavelength (AERP×CV). Within each group, AERP, CV, and wavelength showed significant dependence on cycle length between 100 and 150 ms \(P<0.0001\) in each case, 2-way ANOVA with repeated measures). Data at a cycle length of 75 ms are bracketed to indicate missing data (see text). Sample sizes are as shown in Figure 2C. *P<0.05 and **P<0.01, Bonferroni post hoc test versus Sham at 20 weeks postsurgery. AERP indicates atrial effective refractory period. Other abbreviations as in Figure 3.

Figure 6. Atrial conduction during sinus rhythm. A, Representative examples of conduction maps during sinus rhythm 20 weeks postsurgery (2 examples each of Sham and AoB) (color scale as in Figure 1). B, Index of inhomogeneity. ***P<0.0001, Bonferroni post hoc test versus age-matched control. ###P<0.01, Bonferroni post hoc test 20-week AoB versus earlier AoB. C, Correlation between index of inhomogeneity and duration of AF for 17 hearts in which AF was induced (data from Figure 2C). Abbreviations as in Figure 3.
with the suggestion that impaired filling because of stiffening associated with hypertrophy resulted in increased hemodynamic load on the LA wall. With long-standing elevation of afterload, the LA became hypertrophied, and there was a significant correlation between the LA weight/heart weight ratio and the duration of AF ($r = 0.608, P = 0.010$). Although it is not possible to establish from the present data whether a pathway of sufficient length to accommodate a reentrant circuit existed within the atria of AoB hearts because recordings could not be made from the entire surface of the LA, the data are consistent with atrial enlargement as a causal factor in the increased susceptibility to AF.

Conduction Abnormalities and Remodeling of Cx43

The present findings indicate the involvement of conduction abnormalities in the substrate for arrhythmia. Compared with the corresponding control group, mean vectorial CV was significantly slowed and wavelength of excitation shortened in AoB hearts at 20 weeks postsurgery. The reduced expression of Cx43 may have contributed to the changes in CV, although the possibility that remodeling of other gap junction proteins also may have played a role cannot be entirely excluded. The wavelength of excitation was inversely correlated with the duration of AF (eg, WL$_{100\,\text{ms}}$ $r = -0.593$, $P < 0.05$), which could be interpreted as consistent with a macroreentrant mechanism underlying the arrhythmia. However, the high-resolution mapping of excitation from a very small area ($<1.8\,\text{mm}^2$) of the epicardial surface of the LA free wall of the perfused hearts demonstrated the existence of considerable heterogeneity in excitation during the arrhythmia. Although mapping from a limited area means that it is not possible to be certain of the pathways for excitation during the arrhythmia, these high-resolution data nevertheless demonstrate the existence of multiple wavefronts consistent with a microreentrant mechanism. Moreover, the change in the inhomogeneity of conduction also provides strong evidence that localized conduction abnormalities played an important role in the substrate for arrhythmia, as has been suggested in other models of structural heart disease. 

Consistent with this proposal, the index of inhomogeneity was strongly correlated with the duration of AF (Figure 6C). Thus, the present findings are consistent with the notion of an association among interstitial fibrosis, conduction abnormalities, and AF in rodent hearts. Although reduced expression of Cx43 concomitant with interstitial fibrosis has
been reported in some studies of AF in patients, in other studies, Cx43 expression was found to be unchanged or even increased.26–30 The differences between reports in Cx43 expression may reflect the complex etiology of AF in patients. Nevertheless, the present data support the proposal that the loss of phosphorylated Cx43 protein from the intercalated disks contributes to conduction abnormalities and the substrate for arrhythmia in diseased atria, as has been reported in human AF and in animal models associated with hemodynamic overload of the atrium.26–32

Conclusions
Long-term elevation of afterload in the absence of heart failure in AoB rats resulted in arrhythmogenic remodeling of the LA. Similar to other models of structural heart disease,10–14,28,31 the substrate for arrhythmia involved fibrosis, conduction abnormalities, and remodeling of Cx43 gap junction protein expression. These findings strongly support the notion that elevated ventricular afterload causes atrial remodeling, presumably through increased hemodynamic load on the LA wall.6 The mechanisms underlying the consequent LA remodeling remain unclear but are likely to involve activation of stretch-induced pathways. Further characterization of the signaling pathways underlying the pathogenesis of the arrhythmic substrate in this model has the potential to identify novel targets for the prevention of AF associated with elevated afterload.

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The existence of systemic hypertension and the presence of left ventricular hypertrophy and left atrial dilation on echocardiography are epidemiological predictors of atrial fibrillation (AF). It has been suggested that increased hemodynamic load on the atrial wall results in atrial remodeling associated with atrial enlargement and fibrosis predisposing to AF. However, the etiology of AF is complex, and patients may have multiple risk factors for the arrhythmia, making the study of the pathogenesis of the arrhythmia in patients difficult. Although studies in animal models have established heart failure, mitral valve regurgitation, and long-standing systemic hypertension as causes of proarrhythmic atrial remodeling, surprisingly until now, it has not been clear whether the elevation of left ventricular afterload alone produces a substrate for AF. Using a rat surgical model, this study demonstrates that long-standing pressure overload alone, in the absence of heart failure or systemic hypertension, causes atrial remodeling associated with atrial hypertrophy, enlargement, and fibrosis. This remodeling was associated with an increased inducibility of left atrial fibrillation in isolated, perfused hearts. The reduced expression of connexin43 protein was associated with slowing of conduction velocity and localized conduction abnormalities. The conduction mapping data were consistent with a microreentrant mechanism. The data support the notion that increased hemodynamic load represents an important factor in the pathogenesis of AF. This model may be useful in the future investigation of the mechanisms of atrial remodeling in elevated afterload.
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