Irregular Rhythm Adversely Influences Calcium Handling in Ventricular Myocardium

Implications for the Interaction Between Heart Failure and Atrial Fibrillation

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Background—Despite adequate rate control, the combination of atrial fibrillation with heart failure (HF) has been shown, in a number of studies, to hasten HF progression. In this context, we aimed to test the hypothesis that an irregular ventricular rhythm causes an alteration in ventricular cardiomyocyte excitation–contraction coupling which contributes to the progression of HF.

Methods and Results—We investigated the effects of electrical field stimulation (average frequency 2 Hz) in an irregular versus regular drive train pattern on the expression of calcium-handling genes and proteins in rat ventricular myocytes. The effect of rhythm on intracellular calcium transients was examined using Fura-2AM fluorescence spectroscopy. In conjunction, calcium-handling protein expression was examined in left ventricular samples obtained from end-stage HF patients, in patients with either persistent atrial fibrillation or sinus rhythm. Compared with regularly paced ventricular cardiomyocytes, in cells paced irregularly for 24 hours, there was a significant reduction in the expression of sarcoplasmic reticulum calcium (Ca\(^{2+}\)) ATPase together with reduced serine-16 phosphorylation of phospholamban. These findings were accompanied by a 59% reduction (P<0.01) in the peak Ca\(^{2+}\) transient in irregularly paced myocytes compared with those with regular pacing. Consistent with these observations, we observed a 54% (P<0.05) decrease in sarcoplasmic reticulum Ca\(^{2+}\) ATPase protein expression and an 85% (P<0.01) reduction in the extent of phosphorylation of phospholamban in the left ventricular myocardium of HF patients in atrial fibrillation compared with those in sinus rhythm.

Conclusions—Together, these data demonstrate that ventricular rhythmicity contributes significantly to excitation–contraction coupling by altering the expression and activity of key calcium-handling proteins. These data suggest that control of rhythm may be of benefit in patients with HF. (Circ Heart Fail. 2012;5:786-793.)

Key Words: atrial fibrillation ■ heart failure ■ excitation–contraction coupling ■ calcium-handling proteins ■ cardiomyocytes

ATrial fibrillation (AF) and heart failure (HF) are common cardiovascular disorders which are associated with significant morbidity, mortality, and health expenditure. The 2 conditions are often encountered together in clinical practice, and it has been suggested that their combination is accompanied by particularly adverse outcomes, although this remains controversial. In particular, in several large HF cohorts it has been shown that the presence of AF confers increased risk of HF-related mortality, although other studies have not observed this interaction.

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Although several mechanisms have been proposed to account for the adverse influence of AF on HF, these remain speculative in many cases with little characterization at the molecular level. First, hemodynamic factors, including loss of atrial transport, valvular regurgitation, and persistently irregular ventricular cycles, have been shown to contribute to reduced cardiac output or increased pulmonary capillary wedge pressure independently of heart rate in a range of in vivo models. Second, chronic elevation of heart rate associated with AF may induce tachycardia-mediated cardiomyopathy. Here, the degree of induced left ventricular (LV) dysfunction is related to the rate and duration of tachycardia, and recovery of LV systolic function is expected after restoration of normal sinus rhythm (SR). Subgroup analyses of both retrospective and prospective clinical cohorts indicate that the adverse influence of AF on HF might also be explained by the...
presence of an irregular rhythm per se, based on observations that the reversion to SR in patients with LV dysfunction leads to improved LV function even in subjects with adequate rate control.\textsuperscript{5,20–22} These data suggest that issues beyond rate control and hemodynamic effects may play a larger role in the interaction between AF and HF than generally assumed.

In the present study, we aimed to test the hypothesis that prolonged irregular activation of ventricular cardiomyocytes and the ventricular myocardium leads to a hastening of the molecular remodeling in ventricular myocardium that occurs in HF. In particular, it is well known that major changes in excitation–contraction coupling occur in HF with abnormal calcium cycling and alterations in expression of calcium regulatory proteins including the sarco(endo)plasmic reticulum ATPase 2a pump (SERCA) and phospholamban (PLB).\textsuperscript{21,24} We, therefore, examined the effects of irregular rhythm of the expression of calcium-handling genes and proteins and their functional consequences.

**Methods**

All studies were performed with the approval of the relevant institutional review committees, which included the Human Research Ethics Committee at St Vincent’s Hospital, NSW, Australia; Alfred Medical Research and Education Precinct Animal Ethics Committee; and the University Animal Ethics Committee of the University of Queensland. Investigations confirmed with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004), and the principles outlined in the Declaration of Helsinki.

**Cardiomyocyte Cell Culture and Electrical Field Stimulation**

Neonatal rat ventricular cardiomyocytes (NVCMs) were isolated from day 1 to 2 Sprague-Dawley rat pups by collagenase digestion as previously described.\textsuperscript{25} Cardiomyocytes from each isolation were plated at a density of 1.4×10^5 cells/well on 3 separate 4-well rectangular pacing plates (Nunclon, Nalge Nunc International,). After 24 hours of serum deprivation, cells were paced for 24 hours using a purpose-designed electric field stimulation system (C-Pace EP, IonOptix, Boston, MA). To simulate ventricular activity during normal SR, regular electrical stimulation was delivered at 2 Hz. AF was simulated by irregular electrical stimulation which was generated by a preprogrammed irregular drive sequence derived from an inbuilt repeating sequence of 100 intervals (Figure 1). Using this sequence, the pacing interval was programmed to vary by ±60% around an average of 1000 ms. As such, cardiomyocytes were paced regularly or irregularly are electrically stimulated the same total number of times over the 24-hour period. In all pacing experiments, cardiomyocytes were paced at 20 V using a pulse width of 4 ms and electrical capture was confirmed under light microscopy at the start and end of each pacing period. After 24-hour pacing, cells either underwent protein extraction for further analysis, or were loaded with fura-2AM for measurement of calcium transients. Pacing did not affect cell viability as assessed by Trypan blue (MP Biomedicals, CA) uptake, nor pH of the culture media (data not shown).

**Human Ventricular Myocardial Samples**

LV myocardial samples were obtained from 12 patients with LV ejection fraction <35% undergoing heart transplantation for end-stage HF. We specifically selected tissue samples from stored tissue bank material from 6 patients in SR and 6 patients who were in chronic AF. The cause of HF was nonischemic cardiomyopathy in 6 of 6 and 5 of 6 patients, respectively. Control LV myocardial samples were also obtained from 3 unused donor hearts. The tissue samples were immediately frozen in liquid nitrogen and stored at −80°C. Tissue collection was performed with the approval of the institutional ethics review committees.

**Cell Lysate and Homogenate Preparation**

Human LV homogenates were prepared using a handheld homogenizer (TissueRuptor, Qiagen, MD) in ice-cold phospholysin buffer containing (mmol/L): 10 Tris-HCL (pH 7.5), 50 NaCl, 100 NaF, 10 Na_3P_2O_7, 1 Na_2VO_4, 2 DTT, 1% Triton X-100, and protease inhibitor cocktail (Roche, NSW, Australia). Phospholysis buffer was also applied to NVCMs. Samples were centrifuged at 2500g for 10 minutes and again at 10 000g for 10 minutes to remove debris. Total protein concentration of the supernatant was determined using a protein assay kit (Bio-Rad, CA), with remaining supernatant flash-frozen for storage at −80°C.

**Determination of Protein Expression by Western Immunoblotting**

Ten to 50 μg of protein contained in whole-cell lysates were combined with Laemmli’s sample buffer (Bio-Rad, Hercules, CA), fractionated using sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. As appropriate, blots were probed with primary antibodies recognizing SERCA, total and phosphoserine-16 forms of PLB (t-PLB and p16-PLB), ryanodine receptor (RyR) and sodium-calcium exchanger (NCX). The blots were finally incubated in horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies. All other primary and secondary antibodies were obtained from Affinity Bioreagents (Golden, CO).

**Determination of mRNA Expression Using Real-time Polymerase Chain Reaction**

Total RNA was extracted from human LV homogenates and NVCMs using Trizol reagent (Invitrogen, Carlsbad, CA), purified (Ambion RNA-free Kit, Applied Biosystems, CA), and reverse-transcribed (Taqman Reverse Transcription Reagents, Applied Biosystems). Real-time polymerase chain reaction was performed using SYBR green dye and a 7300 real-time polymerase chain reaction System (Applied Biosystems). The following primers were used: SERCA–forward 5′-GAAGCGCTGGATTGCGA-3′, reverse 5′-GTT TTCCGCAAGCTTGTTGCA-3′; NCX–forward 5′-GCCCTCCC TTTCGATTACGT-3′, reverse 5′-TGGAGACAATGAAACG CC-3′; RyR2–forward 5′-TGGACCATGAAAACCG GC-3′, reverse 5′-AAGCGTGGCCACAGGAT-3′. 18S was used as the housekeeping gene.

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**Figure 1.** Irregular pacing protocol comprising 100 randomly assigned stimulus intervals, with an average interval of 500 ms (2 Hz).
Fluorescence Measurement of NVCM Calcium Transients

After 24 hours of SR pacing, AF pacing, or no pacing in controls, intracellular calcium was determined in NVCMs using microscopy fluorometry under regular 1 Hz pacing conditions using an Ionoptix (Milton, MA) calcium imaging system. In brief, cells were loaded with 5.0 μmol/L fura-2AM and then the media replaced with physiological buffer containing (mmol/L): 139 NaCl, 3 KCl, 17 NaHCO3, 12 Glucose, 3 CaCl2, and 1 MgCl2, pH 7.4. Changes in fluorescent emission at 510 nm recorded with a CCD camera, after alternate excitation at 340 nm and 380 nm, were collected and analyzed using image analysis software. Calcium [Ca2+]i was measured as the ratio of fura-2 fluorescence emission at 340 nm and 380 nm (F340/F380).

Statistical Analyses

Results are expressed as mean±SEM. For tissue culture expression studies, data were derived from 6 independent myocyte preparations. For between-group comparisons, the Student t test was used for normally distributed data and the Mann-Whitney rank-sum test was used for non-normal data. For comparisons of 2 or more groups, 1- or 2-way ANOVA was used, followed by post hoc Tukey multiple comparison tests (for fluorescence imaging analyses) or Bonferroni tests (for molecular biological analyses), respectively, to compare paired means. In all analyses, P<0.05 was taken as significant. Statistical analysis was performed using SPSS (Chicago, IL) version 19 (Cary, NC).

Results

Influence of Electromechanical Rhythmicity on Ca2+ Handling Protein Expression

We first evaluated the effect of irregular ventricular rhythm on the mRNA and protein expression of SERCA, and its key regulatory protein, phospholamban (PLB), given their critical role in determining of the intracellular Ca2+ concentration. These studies were conducted in both human LV tissue and paced rat cardiomyocytes. As shown in Figure 2A and 2C, the expression of SERCA protein expression was significantly lower in ventricular myocardium obtained from patients with HF as compared with healthy control myocardium (56% reduction, P<0.05) and ventricular myocardium obtained from patients with HF in SR (54% reduction, P<0.01). In keeping with this finding, the abundance of SERCA mRNA was significantly lower in failing ventricular myocardium from patients in AF compared with control myocardium (78% reduction, P<0.005) and ventricular myocardium from patients with HF in SR (66% reduction, P<0.005). Similar to observations in human heart, we found a significant reduction in the expression of both SERCA protein (Figure 2B and 2D) and mRNA (19±8%, P<0.05) in irregularly paced ventricular cardiomyocytes, suggesting that rhythm per se does influence SERCA expression beyond any effect of HF.

Using the same approach we demonstrated a significant effect of rhythm on the abundance of phosphorylated PLB in relation to total PLB expression. As shown in Figure 3A and 3C, there was a significant hypophosphorylation of PLB in ventricular samples obtained from patients with HF. More specifically, compared with control samples, the ratio of phosphoserine-16 abundance to total PLB expression was reduced by 60% in SR samples (P<0.05) and by 94% in AF samples (P<0.02), and the extent of PLB phosphorylation was significantly lower in ventricular samples from patients with HF in AF compared with those in SR (P<0.01). In support of these observations, irregularly paced cardiomyocytes exhibited a 57±4% (P<0.05) reduction in the level of PLB phosphorylation (Figure 3B and 3D), compared with regularly paced myocytes. Although the level of PLB phosphorylation was influenced by rhythm, there was no evidence of an effect on the abundance of total PLB protein (Figure 3) or mRNA in either human myocardium or in paced myocytes.

To complement the observations on the expression of SERCA and PLB, we also examined the influence of rhythm on the mRNA and protein expression of the RYR and the NCX in both human tissue and paced rat cardiomyocytes. In failing human LV myocardium from patients in SR, there was a 38% decrease in RYR mRNA expression (P<0.05) compared with that in healthy control tissue. In comparison with tissue from the SR group, the RYR mRNA abundance was decreased significantly further in patients with HF in AF (50%, P<0.05). At the protein level (Figure 4), decreased RYR expression was evident in HF samples compared with controls 1.8±0.3 versus 0.5±0.2 a.u., showing significant RYR expression in patients with HF in AF compared with control myocardium (P<0.05) and ventricular myocardium from patients with HF in SR (66% reduction, P<0.005). Similar to observations in human heart, we found a significant reduction in the expression of both SERCA protein (Figure 2B and 2D) and mRNA (19±8%, P<0.05) in regularly paced ventricular cardiomyocytes, suggesting that rhythm per se does influence SERCA expression beyond any effect of HF.

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but there did not seem to be a between-group difference with regard to rhythm. By contrast, in paced myocytes the abundance of RYR mRNA was increased significantly in irregularly paced cardiomyocytes compared with regular pacing (59 ± 24%, P < 0.05); however, no change in the abundance of RYR protein was evident. In both failing LV myocardium and in paced cardiomyocytes we did not detect any effect of rhythm on the expression of NCX (Figure 4).

Effect of Rhythm on [Ca\textsuperscript{2+}] in Ventricular Myocytes

To determine whether the alterations in the expression of SERCA and phosphorylated PLB associated with altered rhythm resulted in an alteration in intracellular Ca\textsuperscript{2+} handling, we performed calcium imaging rat cardiomyocytes after 24 hours of irregular or regular electrical stimulation. As shown in Figures 5 and 6, the Ca\textsuperscript{2+} transient amplitude was significantly reduced in irregularly paced NVCMs compared with regularly paced or unpaced NVCMs (59% and 82%, respectively, P < 0.0001). By detailed analysis of the Ca\textsuperscript{2+} transient we also observed that the time to 10%, 50%, and 90% of peak calcium from baseline was prolonged in AF versus SR paced NVCMs (Figure 5B), whereas the rate of calcium decay reflected by time to 10%, 50%, and 90% return to baseline did not seem to change significantly across groups (Figure 5C).

Discussion

The present study sought to establish whether the clinically observed adverse association between AF and HF could be explained by a deleterious effect of irregular electromechanical activity on ventricular cardiomyocytes. To the best of our knowledge, we showed for the first time that irregular stimulation of ventricular cardiomyocytes, to simulate the ventricular activity in AF, is associated with a significant downregulation of SERCA and in the ratio of phosphorylated to total PLB. These changes were associated with significant alterations in the regulation of intracellular calcium levels.

As outlined above, we sought to specifically distinguish the impact of rhythmicity on cardiomyocyte function, separate to the effects of rate in poorly controlled AF. Previous work by Lenaerts et al.\textsuperscript{26} evaluated mechanical function, Ca\textsuperscript{2+} handling, and cell structure in atrial myocytes obtained from sheep with AF induced by rapid atrial pacing. Consistent with our study, they showed that AF caused a significant reduction in the amplitude of the Ca\textsuperscript{2+} transient, which was accompanied by reduced contractility. In contrast to our study, however, this was proposed to be the result of an altered coupling between the L-type Ca\textsuperscript{2+} channel and the RYR. Of note, the present study differed in several respects to that of Lenaerts, including the effect of differences in rate per se between myocytes obtained from sheep in SR to those in AF and the use of atrial myocytes which express ion channels in different proportions to that of ventricular cardiomyocytes.\textsuperscript{26} In the present study, we did not measure cardiomyocyte contraction because of the difficulties associated with measuring true cell shortening in neonatal cardiomyocytes.

Previous studies of SERCA abundance in failing human LV myocardium have generally shown reduced mRNA\textsuperscript{27} and protein\textsuperscript{28–30} expression compared with nonfailing hearts. A clear relationship between SERCA protein abundance or activity and contractile function has been demonstrated in some, although not all studies.\textsuperscript{30} HF has also been associated...
with reduced serine-16 phosphorylation of PLB. Together, these changes contribute to reduced SERCA activity and SER calcium loading, diminished calcium-induced calcium release, and consequently depressed contractile response. Reduced SERCA activity also results in diastolic impairment because of delayed elimination of cytosolic calcium after contraction; although in the current study we did not detect a prolongation of the time to restoration of basal intracellular calcium levels. We could not also attribute this to enhanced expression of NCX as suggested by others.

In our study, we did not observe a significant difference in SERCA protein expression between failing myocardium from patients in SR and nonfailing LV myocardium. Of note, this is consistent with several negative studies, with larger positive studies recognizing a wide range of SERCA expression to exist in LV myocardium from both nonfailing and failing hearts of up to 2-fold and 4-fold, respectively. Our findings suggest that consideration of the underlying heart rhythm may also be of relevance in the interpretation of some of these previous studies. Although the role of SERCA has been extensively studied in HF, the cellular mechanisms responsible for the changes in expression are largely unknown. Some indirect evidence suggests that calcium may influence the expression of SERCA via an incompletely understood nuclear factor of activated T cells-mediated mechanism.

In addition to the level of SERCA protein expression, the corresponding level of PLB expression and its relative level of phosphorylation is also a critical determinant of SERCA activity and thus intracellular calcium homeostasis. We found that in both the failing human heart and in our cell culture model, the presence of an irregular rhythm was accompanied by a significant reduction in the ratio of phosphorylated to total PLB, whereas the overall level of PLB was unchanged. The notion that the relative hypophosphorylation of PLB could contribute to a reduction in ventricular function is supported by studies in which a pseudophosphorylated form of PLB was introduced into isolated cardiomyocytes or the intact heart, demonstrating an improvement in ventricular function in the setting of experimental HF. Phosphorylation of PLB is well understood to be regulated via the influence of protein kinase A, typically under the activation of β adrenergic receptors. Conversely, the dephosphorylation of PLB is mediated via protein phosphatase 1, which has also been implicated in the pathogenesis in HF, although its role in AF is unknown. Furthermore, in the setting of HF, it has been previously shown that reduced levels of inhibitor-1 protein together with its degree of phosphorylation may account for the increased activity of protein phosphatase 1 and thus diminished PLB phosphorylation. As a functional correlate of our observations in paced myocytes, we found that there was a marked reduction in the peak intracellular calcium concentration in comparison with regularly paced ventricular myocytes. This would be consistent with the depletion of SER calcium in the setting of reduced SERCA activity, and would be expected to translate into reduced contractility.

In the present study, we found a reduction in the expression of RYR protein and mRNA in failing myocardium, an effect that was further enhanced in the presence of AF. To date, studies of RYR expression have not yielded uniformly consistent findings in human HF. In addition to the expression of RYR per se, several extensive studies also highlight the role of posttranslation modifications of RYR, including hyperphosphorylation, nitrosylation, and oxidation, together with the role of regulatory protein–protein interactions in determining the open probability of the RYR channel (as reviewed recently). Although point mutations involving RYR and possibly phosphorylation of RYR may increase Ca²⁺ through RYR channels contributing to the generation of ventricular arrhythmias, it is not certain whether this process also reduced cardiac contractility.
study, we did not investigate the effect of rhythm on RYR phosphorylation, and the sample size was too small to discern a specific effect of concomitant pharmacologic therapies.

Although our studies in cardiomyocytes were performed in rat neonatal cardiomyocytes, these cells are frequently used for studies of gene and protein regulation. Our study was designed specifically to compare the differential effect of irregular versus regular electromechanical activity on gene and protein expression, together with calcium handling, although it is known that some differences in excitation–contraction coupling exist between neonatal and adult cardiomyocytes. In the present study, we did not investigate the effects of rhythmicity on ion channel activity or cell–cell communication. Notwithstanding the potential limitations of neonatal myocytes, our cellular studies were consistent with those found in studies on human myocardium.

As a corollary of our study it might be predicted that the conversion of patients with HF in AF to SR could be accompanied by evidence of improved ventricular function. Indeed, van Gelder et al previously showed that cardioversion from AF caused a progressive rise in LV ejection fraction during a 1-month period. Moreover, the increase in LV function did not track changes in atrial mechanical function, leading to the conclusion that AF induced a cardiomyopathic process.

Subsequently, several clinical studies have been conducted to investigate the effect of rhythm versus rate control on survival and ventricular function in patients with AF. In the AFFIRM study, there were no differences in mortality between the 2 management strategies. This study was limited by the presence of relatively few patients with HF and low success in SR maintenance. Of note, a further analysis of the AFFIRM study did show that the presence of SR was associated with better survival. In the RACE study, Hagens and colleagues compared the effects of rate versus rhythm-control strategies in HF patients with AF. Similar to the Atrial Fibrillation Follow-up Investigation of Rhythm Management (AFFIRM) study, this study failed to demonstrate a difference between the 2 treatment groups; however, the study was also limited by the fact that only 36% of patients in the rhythm-control group were in SR at follow-up. Interestingly, in that study, a multivariate analysis suggested that the presence of SR was associated with improved LV function.

More recently, a large-scale multicenter trial compared rate versus rhythm control in patients with more advanced HF. This study demonstrated that both treatment strategies were equal with regard to survival and HF hospitalization. Although this study does not necessarily support the hypothesis that AF leads to an acceleration of HF progress, it is important to note that these patients did not have sustained AF at recruitment, and during follow-up there was heterogeneity of rhythm across both treatment groups. This issue also confounds the recent Registry on Cardiac Rhythm Disorders Assessing the Control of Atrial Fibrillation (RECORDAF) study, which showed that although patients with HF receiving a rhythm management strategy had fewer hospitalizations, the interpretation of the study is complicated by the limit success of the rate and rhythm strategies and the higher baseline incidence of HF in the rate control group.

Given the major limitations of pharmacologic strategies for rhythm control, other investigators have used more definitive electrophysiologic approaches. Ferreira et al showed that AV node ablation in conjunction with biventricular pacing resulted in improved survival and reduced hospitalization compared with patients in AF with a biventricular pacemaker. This study is supported by a previous meta-analysis which showed that AV node ablation improved ventricular function, which together are consistent with the present study.

Limitations

Our study has several limitations. The study made use of human tissues obtained from transplant recipients and unused donor hearts. Detailed information regarding the use and doses of inotropic therapy, antiarrhythmics, β-blockers, and digoxin was not available in all patients. Accordingly, it was not possible to match in detail AF and non-AF patients. In the present study, we studied and compared the effect of regular versus irregular electromechanical activity on Ca²⁺ handling and Ca²⁺ handling protein expression in neonatal cardiomyocytes to understand the potential effects of AF in HF. These studies were of a short-term nature, and they may not necessarily reflect the influence of persistent or permanent AF. We did not specifically study the effects on cardiomyocyte contractility and did not evaluate to effects on other intracellular signaling pathways which may also affect contractility.

Taken together, our study demonstrates in ventricular cardiomyocytes and intact myocardium that irregular cycles of excitation and contraction induce an altered profile of gene and protein expression that are associated with functional changes at a cellular and tissue level. These data were derived from series of consistent studies performed using complimentary approaches that include isolated cardiomyocyte and explanted human heart samples. These observations may provide an explanation for the deleterious interaction between AF and HF. Our study provides support for continued investigation into the effect of strategies that effectively restore regular ventricular rhythm in HF patients with AF.

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Disclosures

None.

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**CLINICAL PERSPECTIVE**

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