Arrhythmogenic Remodeling of $\beta_2$ Versus $\beta_1$
Adrenergic Signaling in the Human Failing Heart

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Background—Arrhythmia is the major cause of death in patients with heart failure, for which $\beta$-adrenergic receptor blockers are a mainstay therapy. But the role of $\beta$-adrenergic signaling in electrophysiology and arrhythmias has never been studied in human ventricles.

Methods and Results—We used optical imaging of action potentials and [Ca$^{2+}$]$_i$ transients to compare the $\beta_1$- and $\beta_2$-adrenergic responses in left ventricular wedge preparations of human donor and failing hearts. $\beta_1$-Stimulation significantly increased conduction velocity, shortened action potential duration, and [Ca$^{2+}$]$_i$ transients duration (CaD) in donor but not in failing hearts, because of desensitization of $\beta_1$-adrenergic receptor in heart failure. In contrast, $\beta_2$-stimulation increased conduction velocity in both donor and failing hearts but shortened action potential duration only in failing hearts. $\beta_2$-Stimulation also affected transmural heterogeneity in action potential duration but not in [Ca$^{2+}$]$_i$ transients duration. Both $\beta_1$- and $\beta_2$-stimulation augmented the vulnerability and frequency of ectopic activity and enhanced substrates for ventricular tachycardia in failing, but not in donor, hearts. Both $\beta_1$- and $\beta_2$-stimulation enhanced Purkinje fiber automaticity, whereas only $\beta_2$-stimulation promoted Ca-mediated premature ventricular contractions in heart failure.

Conclusions—During end-stage heart failure, $\beta_2$-stimulation creates arrhythmogenic substrates via conduction velocity regulation and transmurally heterogeneous repolarization. $\beta_2$-Stimulation is, therefore, more arrhythmogenic than $\beta_1$-stimulation. In particular, $\beta_2$-stimulation increases the transmural difference between [Ca$^{2+}$]$_i$, transients duration and action potential duration, which facilitates the formation of delayed afterdepolarizations.

Key Words: arrhythmia (mechanisms) ■ calcium ■ heart failure ■ receptors, adrenergic

Increased $\beta$-adrenergic sympathetic activity is a hallmark of heart failure and often leads to ventricular remodeling and impaired cardiac contractile function. Up to 50% of heart failure, mortality is sudden because of arrhythmias, $\beta_1$- or nonselective adrenergic blockers can reverse ventricular remodeling and reduce mortality in patients with heart failure. Although the antiarrhythmic effect of $\beta$-blockers has long been recognized,
the underlying mechanism is incompletely understood. To date, the effect of $\beta$-adrenergic signaling on electrophysiology and arrhythmias has never been studied in tissue preparations from failing and nonfailing human hearts.

$\beta$-Adrenergic stimulation can lead to sarcoplasmic reticulum Ca overload, spontaneous sarcoplasmic reticulum Ca release, and delayed afterdepolarization (DAD). In addition, $\beta$-receptors regulate multiple ion channels via the cAMP/protein kinase A (cAMP/PKA) cascade, which is electrogenic and potentially arrhythmogenic. However, cellular studies cannot fully explain arrhythmogenesis and arrhythmia maintenance at the tissue level. Potential arrhythmogenic substrates because of $\beta$-stimulation include abnormal impulse conduction, altered repolarization dynamics, and transmural heterogeneity in repolarization. The human ventricular free wall is composed of 3 layers: subendocardium, midmyocardium, and subepicardium. These layers possess different intrinsic electric properties that account for activation delays and synchronized ventricular repolarization. In heart failure, these properties are altered because of molecular and structural remodeling, including remodeling of $\beta$-adrenergic receptors (ARs), and may set the stage for triggered arrhythmias, as seen in patients with heart failure.

Two dominant subtypes of $\beta$-AR are $\beta_1$- and $\beta_2$-AR. Although $\beta_1$-AR predominates in the human myocardium, $\beta_2$-AR is equally important because of its more efficient adenylyl cyclase coupling and endogenous role in cardiac regulation. Recently, the use of nonselective $\beta_1$- or $\beta_2$-blockers was advocated by clinical trials and studies, emphasizing the importance of $\beta_2$-adrenergic signaling in heart failure. Although the effects of remodeling the $\beta_1$/$\beta_2$-adrenergic system on...
WHAT IS KNOWN

- Excessive β-adrenergic stimulation is a hallmark of heart failure, which is associated with ventricular arrhythmias, a major source of death.
- However, the arrhythmogenic mechanisms of β-adrenergic signaling have never been studied in the human failing and nonfailing left ventricle.

WHAT THE STUDY ADDS

- In this study, we investigated the arrhythmogenic substrates resulting from β₁ and β₂-adrenergic stimulation during end-stage human heart failure and found that β₂-stimulation, but not β₁-stimulation, increases arrhythmia vulnerability through effects on both arrhythmogenic triggers and substrates.
- The findings suggest that targeting β₂-adrenergic pathway may be more effective in treating and preventing arrhythmogenesis in heart failure than traditional β₁-selective blockers.

contractility have been studied in human trabeculae, the effect of β₁/β₂-remodeling on electrophysiology and arrhythmogenesis in the left ventricular (LV) wall of the human failing heart is unknown.

We hypothesized that in end-stage heart failure, β₁- and β₂-adrenergic signaling are differentially remodeled and have different effects on arrhythmogenesis. To compare β₁- and β₂-selective adrenergic effects, we assessed excitation–contraction coupling using dual optical mapping of action potential (AP) and [Ca²⁺] transients (CaT) in LV wedge preparations from human failing and donor hearts. Our data indicate that β₂-stimulation, in contrast to β₁-stimulation, induces transmural heterogeneity in repolarization and sets the stage for arrhythmias. Furthermore, we show that β₂-AR signaling enables the formation of DADs by increasing the difference between CaT duration (CaD) and AP duration (APD), suggesting an underlying mechanism of triggered arrhythmias, such as those observed in patients with heart failure.

Material and Methods

Experimental Protocol

Procurement and use of human myocardium were approved by the Institutional Review Board of Washington University School of Medicine. Nonischemic end-stage failing human hearts (n=8) were procured at the time of transplantation at Barnes-Jewish Hospital. Nonfailing donor hearts (n=8) were procured at Mid-America Transplant Services (St Louis, MO) and were used as controls. The clinical data of patients are shown in Table I in the Data Supplement.

The arterially perfused LV wedge preparations (Figure I in the Data Supplement) and dual optical mapping were described previously10,11 and in Methods in the Data Supplement. Fluorescent probes, RH237 and Rhod-2-AM, were used for AP and CaT, respectively. We used selective β₁-AR agonist procaterol (1 μmol/L),12 selective β₂-AR agonist xamoterol (1 μmol/L),13 and nonspecific β-AR agonist isoproterenol (100 nmol/L; Tocris Bioscience, Ellisville, MO; Figure II in the Data Supplement). The agonists were applied at saturating concentrations for maximum effective activation of the receptors.10,13 Complete washout evaluated by the recovery of conduction velocity (CV), APD, and CaD was applied between xamoterol and procaterol.

Statistical Analysis

For studies in which baseline and β-stimulation measures were compared, we conducted analyses with a mixed random effects repeated measures model with group, time, and group-by-time interaction as fixed effects. For each analysis, several covariance structures were fit, and the fit with the smallest Bayesian Information Criteria score was used, which was the Toeplitz matrix structure. For experiments in which transmural layers were analyzed separately, we used a mixed random effects split-plot study design to account for layers having different degrees of freedom relative to the group. Post hoc t tests were then performed by Bonferroni correction. We considered P<0.05/(number of pairwise tests) to be statistically significant.

In Methods in the Data Supplement, we provide detailed data analysis and describe experimental protocols and methods, including Western blot, quantitative polymerase chain reaction, and immunofluorescence.

Results

Effects of β₂ Versus β₁-AR Stimulation on Arrhythmia Substrates in Heart Failure

Effects of β₂-AR Stimulation on CV

We quantified anisotropy as the ratio between longitudinal conduction velocity (CV_L)/transverse conduction velocity (CV_T; Figure 1).14 In both donor and failing hearts, CV_L did not demonstrate a physiologically significant difference after β₁- or β₂-stimulation, although there was a statistically significant difference for β₂-stimulation in failure (Figure 1C and 1D). Both β₁- and β₂-agonists dramatically increased CV_L in donor hearts. In failing hearts, only β₂, but not β₁, agonist increased CV_L (Figure 1E and 1F). The quantitative analysis of velocity changes is shown in Figure III in the Data Supplement. Accordingly, anisotropy decreased with β₂-stimulation and increased with β₁-stimulation in failing hearts; however, in donor hearts, only conduction anisotropy is increased significantly during β₂-stimulation (Figure 1G and 1H).

β₂-Stimulation Increased Transmural Heterogeneity of Repolarization in Failing Hearts

To assess the effect of β₂-stimulation on repolarization, we quantified APD at 80% of repolarization in the subendocardium (endo), midmyocardium (mid), and subepicardium (epi; Figure 2). Classification of all layers is shown in Figure II in the Data Supplement. As previous studies have also shown,15 we determined that APD in failing hearts, measured at different pacing rates in the absence or presence of β₂-stimulation, is significantly longer than APD in donor hearts (Figure 2B). Both β-agonists shortened APD in both groups. However, for the different layers, the agonists reduced APD to varying degrees (Figure 2C; Table II in the Data Supplement). This layer-dependent APD reduction increased the APD difference between endocardium and epicardium (APD_endocardium−APD_epi) dramatically more in failing hearts than in donor hearts (Figure 2D).
We quantified the layer-dependent APD reduction (ΔAPD) at 1 Hz pacing in response to both β-agonists as the difference of APD values between baseline and in the presence of β-agonists (Figure 3A). ΔAPD maps revealed that β1- and β2-stimulation each resulted in different spatial patterns of APD reduction (Figure 3B).

In donor hearts, β2-agonist stimulation caused decreasing ΔAPDs (increasing reductions in APD) from the epicardium toward the endocardium. The opposite is seen in failing hearts, where β2-agonist treatment caused increasing ΔAPDs (decreasing reductions in APD) from the endocardium to the epicardium (Figure 3C). Taken together, the ΔAPD effect sizes between donor and failing hearts were greatest at the epicardium and decreased toward the endocardium. Strongly significant group-layer interactions for ΔAPD values (P<0.0001) highlight the differences in effect sizes between donor and failing hearts (Figure 3C). In failing hearts, the average APD reduction caused by β2-agonist in the transmural wall is greater than that in donor hearts. This suggests that the β2-AR pathway is sensitized in heart failure. β1-Agonist treatment produced APD reductions that were more pronounced in the endocardium and decreased toward the epicardium of donor hearts. In failing hearts, a modest and uniform APD reduction was observed. In donor hearts, β1-agonist induced a significant APD reduction in all layers. This demonstrates that the β1-AR pathway is desensitized in failing hearts (Figure 3D).

We aimed to determine the molecular mechanism underlying the layer-dependency of β2-stimulation by quantifying β2-AR protein expression. In contrast with previous findings,16 we observed that, in donor hearts, the expression of β2-ARs was higher in the endocardium than in the epicardium, with a β2-AR-epi/β2-AR-endo ratio <1. In failing hearts, the expression of β2-ARs was reversed: higher expression is observed in the epicardium than in the endocardium (Figure 3E), with a β2-AR-epi/β2-AR-endo ratio >1. The reversed transmural gradients in β2-AR protein expression may explain the reversed endocardial-to-epicardial gradient effect of β2-stimulation on APD in failing hearts.

Figure 1. Effects of β-stimulation on conduction velocity (CV) and anisotropy. A, Activation map illustrates CV measurements as a function of direction. B, Averaged wavefronts at each condition from donor and failing hearts are shown. Longitudinal conduction velocity (CVL) values for (C) donor and (D) failing hearts are shown compared with baseline values before stimulation. Graphs of transverse conduction velocity (CVT) for (E) donor and (F) failing hearts with both stimulation conditions compared with baseline values. G, Donor and (H) failing heart conduction anisotropy values. β1-Stimulation increased anisotropy in donor hearts but decreased it in failing hearts, whereas β2-stimulation increased anisotropy in failing hearts. Box outlines show the 25th and 75th percentiles, and the middle lines indicate median values. Whiskers show the 5th and 95th percentiles.
Phosphorylation of $\beta_2$-AR by PKA Was Reduced in Failing Human Hearts

$\beta$-Stimulation controls electrophysiological targets primarily via the stimulatory G-protein (G_s) and downstream PKA. Unlike $\beta_1$-AR, which couples only to G_s, $\beta_2$-AR can couple either to G_s or to inhibitory G-protein (G_i). Phosphorylation of $\beta_2$-AR by PKA can switch its coupling from G_s to G_i. Therefore, we assessed $\beta_2$-AR phosphorylation at PKA-phosphorylation site Ser345/Ser346 and found $\beta_2$-AR phosphorylation was significantly lower in failing than that in donor hearts (Figure 3F), suggesting greater coupling to G_i in failure.

Effect of $\beta_2$-AR Stimulation on CaD Shortening

We recorded CaT simultaneously with AP to generate CaD maps (Figure 4A and 4B). CaD values at 80% of Ca relaxation are shown in Figure IV in the Data Supplement. CaD shortening ($\Delta$CaD) was quantified to evaluate the effects of $\beta$-stimulation on Ca reuptake. $\beta_1$-Agonist treatment induced significantly greater CaD reductions in donor than in failing hearts across the transmural wall (Figure 4D; the effect size between donor and failing hearts was transmurally homogeneous, as the group effect was strongly significant ($P=0.0007$), whereas the group-layer interaction was not significant ($P=0.66$). In contrast, $\beta_2$-agonist treatment shortened CaD to
a similar degree in both donor and failing hearts (Figure 4C). Interestingly, stimulation with the β₂-agonist did not induce similar transmural spatial differences as those observed with APD reduction.

**Transmural Gradient of CaD–APD**
The CaD–APD is a measure of the period during which the intracellular calcium concentration ([Ca²⁺]i) remains at a high level after the membrane has been completely repolarized (Figure 4E). Prolongation of this period, defined as Δ(CaD–APD)=(CaD–APD)baseline−(CaD–APD)β₂-stimulation, could lead to a window where the [Ca²⁺]i is higher after β₂-stimulation than at baseline (green arrows in Figure 4E) during the period of complete membrane repolarization. In this vulnerable window, DADs may occur because of inward currents from the Na–Ca exchanger (NCX) while it extrudes Ca²⁺. In failing hearts, we observed prolongation of the Δ(CaD–APD) after β₂-stimulation in the epicardium (−42.8±10.7 ms for donor versus 13.6±6.9 ms for failing; P=0.003) and midmyocardium (−32.0±10.1 ms for donor versus 7.1±7.2 ms for failing; P=0.0074), but not in the endocardium (−23.1±8.0 ms for donor versus −31.6±12.1 ms for failing; P=0.53), where negative values indicates that CaD–APD value after stimulation is shorter than baseline condition. In donor hearts, prolongation of Δ(CaD–APD) after β₂-stimulation was not observed (Figure 4F). Also, there were no significant differences in transmural Δ(CaD–APD)s in either donor or failing hearts after β₁-stimulation (Figure 4G).

**β₂-AR Stimulation Altered CaT Morphology**
To evaluate changes in CaT morphology, we quantified the CaD₃₀/CaD₈₀ ratio. We observed that the ratio is greater at baseline in failing hearts than in donor hearts throughout the transmural wall, as previous findings have also shown (Figure 5A). Treating with β₂-agonist equalized the ratio (donor versus failing, 0.55±0.02 versus 0.55±0.01; ns after β₂-stimulation and 0.52±0.015 versus 0.59±0.009; P=0.003 at baseline), which indicates a change in CaT morphology.
The Ca2+ extrusion was analyzed by determining 2 phases: the early phase (fast Ca2+ decline component), as determined by the Ca2+ decay constant. The T50 average for all layers decreased after β1-stimulation in both donor and failing hearts (Figure 5G). However, the Ca2+ decay constant was similar before and after β1-stimulation in failing hearts (Figure 5H), which suggests that abrogation of β1-regulation of Ca2+ handling occurred mainly during the slow Ca2+ reuptake phase.

β-AR Stimulation Is Necessary for Ectopic Activity in Human Heart Failure

We counted premature ventricular contractions (PVCs) during 8 minutes of pacing (4 minutes of 0.5 Hz and 4 minutes of 0.67 Hz) and automaticity after pacing. We did not observe spontaneous automaticity or PVCs under any condition in donor hearts. In contrast, failing hearts exhibited both ectopic PVCs and automaticity in response to β-stimulation. Multiple types of PVCs were observed after β1-stimulation. Figure 6A shows automaticity and PVCs with different morphologies originating from different locations recorded after β1-stimulation in failing hearts. PVC#1 represents a type of irregularly occurring PVC with small amplitude and slow upstroke that propagated through a limited area, as circled, before being terminated. According to the AP signal amplitude map, these PVCs originated from the midmyocardium. PVC#2 represents another type of PVCs with greater amplitude and fast upstroke that can propagate through the transmural surface. This type of PVC originated from the endocardium (Figure 6A).

Next, we analyzed the underlying mechanisms and involvement of calcium overload in these 2 types of PVCs (#1 and #2). We used AP-CaT delay map to measure the time difference between AP activation and Ca release. Figure 6B shows a PVC#1 where the Ca release occurred before AP upstroke, which suggests that it was triggered by Ca release. Thus, the mechanism responsible for PVC#1 may be a Ca-driven process enhanced by β1-stimulation. In all hearts, Ca-driven PVCs only originated from the subepicardium and midmyocardium (Figure 6B), which were the transmural regions with prolonged CaD-APD.

PVC#2 shared the same origin with a series of regular beats of enhanced automaticity. This automaticity originated from the endocardium with a rate of 33 beats per minute and was eliminated by rapid delivery of Lugol’s solution to the sub-endocardial surface, suggesting that these events are because of the Purkinje fiber automaticity. To test this hypothesis, we determined the presence of the Purkinje fiber marker connexin 40 by immunofluorescence. Figure 6D (top-right) shows that the tissue from where the regular beats originated has high expression of connexin 40 at the intercalated disk, further implicating that Purkinje fiber automaticity underlies the observed events.

β-AR Stimulation Increased Occurrence of Spontaneous PVCs and Rapid Pacing-Induced Ventricular Tachycardia in Failing Hearts

To confirm the described mechanisms of automaticity and ectopy, we used real-time pseudo-ECG and monophasic AP recordings to measure the occurrences of PVCs and pacing-induced ventricular tachycardia (VT; Figure 7A). The number of PVCs and VTs dramatically increased after β-AR stimulation in failing hearts, but neither could be induced in donor hearts (Figure 7C–7E). Both β1- and β2-stimulation induced

Figure 4. Effects of β-stimulation on [Ca2+]i transients (CaT) and CaT duration (CaD)–action potential duration (APD). A, Representative CaT signals. B, Representative CaD maps. C and D, Statistical analysis of CaD shortening (ΔCaD) reveals partial desensitization of β1-adrenergic receptor (AR) but not significant change of β1-AR regulation in heart failure. E, Calculations of Δ(CaD–APD) are shown. F and G, Statistical analyses of Δ(CaD–APD) are shown.

(Figure 5B and 5E). Although the ratio in failing hearts was still greater than in donor hearts after treatment with the β1-agonist, transmural heterogeneity was eliminated in both groups (Figure 5C and 5F); this is demonstrated by a strongly significant group-layer interaction at baseline (P<0.0001), but the absence of a significant group-layer interaction after treatment with β1-agonist (P=0.076).

We analyzed Ca2+ release and extrusion to determine the period in which β1-stimulation causes changes in CaT morphology. We did not observe a difference in Ca2+ release when evaluating the rise time of CaT (Figure V in the Data Supplement). The Ca2+ extrusion was analyzed by determining 2 phases: the early phase (fast Ca2+ decline component), as determined by T50 (period of time from CaT peak to 50% reuptake), and the late phase (slow Ca2+ decline component), as determined by the Ca2+ decay constant. The T50 average for all layers decreased after β1-stimulation in both donor and failing hearts (Figure 5G). However, the Ca2+ decay constant was similar before and after β1-stimulation in failing hearts (Figure 5H), which suggests that abrogation of β1-regulation of Ca2+ handling occurred mainly during the slow Ca2+ reuptake phase.
more automaticity and PVCs in the endocardium when compared with other regions, which mainly originated in the Purkinje fibers. Unlike β₁-agonist, which induced automaticity in the Purkinje fibers only, β₂-agonist also induced PVCs in epicardium and midmyocardium in addition to endocardium, among which >44% were Ca-mediated. No Ca-driven PVCs were observed during β₁-stimulation (Figure 7B).

Discussion

Our data show, for the first time, a differential role of β₁- and β₂-AR pathways in arrhythmogenesis in human heart failure. We show that in failing hearts, β₁- and β₂-stimulation provoke automaticity and PVCs through ≥2 mechanisms. Both β-ARs increase automaticity in Purkinje fibers, but only β₂-AR stimulation causes Ca-driven PVCs. β₂-Stimulation further enhances arrhythmogenic substrates via CV regulation, spatially heterogeneous repolarization, and CaD. Importantly, β₂-stimulation increased the transmural difference between CaD and APD, which facilitated the formation of DAD (summarized in Figure 8B).

Arrhythmogenesis Because of β₂- and β₁-Stimulation During End-Stage Heart Failure

Desensitization of β₁-AR and preserved effects of β₂-AR have been previously reported in gene/protein expression, and cardiac mechanics in human endocardial biopsies from patients with heart failure. We mapped the entire transmural LV and found sensitization of β₂-AR at the subendocardium and midmyocardium of failing hearts. Moreover, in contrast to attenuated β₁-regulation in failing hearts, β₂-regulation is not only sensitized but also remodeled at the molecular and tissue level. This remodeling develops an arrhythmic substrate involving conduction, repolarization, and Ca handling in end-stage heart failure. Although β₂-stimulation affected CV similarly in failing and donor hearts, the average anisotropy was increased only in failing hearts. Because of this increase, the impulse propagates slowly in the transverse direction but rapidly in the longitudinal direction, which may lead to conduction block, whereas β₁-stimulation lowers such vulnerability by decreasing the anisotropy. In addition, β₂-stimulation (but not β₁-stimulation) induced remodeling of repolarization in time and space domains, such as altered refractoriness and increased transmural heterogeneity, which would widen the vulnerability window and facilitate unidirectional block and reentrant arrhythmias. The additional arrhythmogenicity resulting from β₂-stimulation in the human failing myocardium provides mechanism for recent studies advocating the use of nonselective β-blockers or β₂-specific blockers for heart failure.

Switching of β₂-AR Coupling From Gᵢ to Gₛ in Failing Hearts

β₂-AR stimulation resulted in significant reduction of APD across the transmural surface in failing hearts when compared with that in donor hearts, which suggests sensitization of β₂-AR in heart failure. This effect may result from a β₂-AR coupling switch from Gᵢ to Gₛ because of β₂-AR phosphorylation by PKA, which is also activated by Gₛ. We observed significantly reduced β₂-AR phosphorylation in failing hearts, which suggests that β₂-ARs switch from Gᵢ to Gₛ.
in heart failure. The switch from the protective $G_i$-dependent negative feedback control system to an unstable $G_s$-dependent positive feedback control system was rendered via remodeling of $\beta_2$-AR signaling (Figure 8A) in heart failure. Therefore, $\beta_2$-AR could enhance its control of excitation–contraction coupling through switching from $G_i$ to $G_s$, as evident from enhanced $\Delta APD$ in failing hearts. Our observations are in agreement with studies by Gong et al. 23 Their data showed a negative inotropic effect in failing human ventricular cardiomyocytes after application of $\beta_2$-AR antagonist ICI118551. In contrast, cardiomyocytes from nonfailing hearts showed no significant decreased inotropic effect in the presence of $\beta_2$-AR antagonist. These data further support our conclusion that, in heart failure, a greater proportion of $\beta_2$-AR couples with $G_s$. Furthermore, $\beta_2$-AR overexpression has a negative inotropic effect after treatment with ICI118551 in mouse models. 24 An increased proportion of $\beta_2$-AR-$G_s$ coupling was reported in this transgenic mouse model, 24 which, along with previously described work in human hearts,23 lends support to our theory.

$G_i$ to $G_s$ Switch Abrogates Increase of NCX Activity Caused by $\beta_2$-Stimulation

In this study, we established that abrogation of $\beta_2$-regulation during the late phase of Ca extrusion accounted for CaT

Figure 6. Multiple mechanisms of automaticity and premature ventricular contraction (PVC) in heart failure. A, Multiple types of PVCs were observed after $\beta_2$-stimulation. Action potential (AP) amplitude maps were constructed at T1 and T2 time points to determine the location of the origination of both types of PVCs. B, Ca-driven PVC was confirmed by AP-$[Ca^{2+}]$ transients (CaT) delay map and found to share the same location with PVC#1. Activation times from 2 locations were labeled: PVC origination (point #1 and 3 on AP and CaT activation map, respectively) and an adjacent location (#2 and 4). Activation time of 3 (CaT, 22 ms) is earlier than 1 (AP, 26 ms), and this difference is reversed shortly after AP propagated: activation time of 2 (AP, 28 ms) is before 4 (CaT, 30 ms). C, Enhanced automaticity shared the same location as PVC#2. D, Immunofluorescence staining of connexin 40 and 4',6-diamidino-2-phenylindole. Connexin 40 staining was observed in coronary vascular endothelium (top-left) and endocardium (middle-right). The staining is negative in epicardium (bottom-left). The region of enhanced automaticity (small yellow box) expressed connexin 40 (top-right). Intramural tract branch of Purkinje fibers penetrating into myocardium was also observed (bottom-right). AP indicates action potential; endo, subendocardium; epi, subepicardium; FOV, field of view; MAP, monophasic AP; and mid, midmyocardium.
morphological change in heart failure. Between the major proteins (SERCA [sarco/endoplasmic reticulum Ca\(^2\)-ATPase] and NCX) that determine Ca extrusion, NCX has been recognized to contribute more during CaT late phase\(^25\) because of membrane potential being lower than the equilibrium potential of NCX (ENa/Ca).\(^26\) When NCX is overexpressed, \(\beta_2\)-stimulation upregulates its activity through \(\beta_2\)-AR coupling to Gi protein.\(^27\) However, this increase is not supported by our result (Figure 5G).

Given that NCX is overexpressed in heart failure (Figure VI in the Data Supplement), we postulate that reduced \(\beta_2\)-regulation may be because of decreased \(\beta_2\)-AR-Gi coupling.

**Transmural Remodeling of \(\beta_2\)-AR Regulation and DAD**

In contrast to APD regulation, \(\beta_2\)-AR system neither enhanced Ca handling in failing hearts nor increased transmural CaD heterogeneity in either group. This disparity resulted in increased CaD–APD difference, which exacerbated DAD initiation. Here, we show DAD triggered PVCs associated with Ca release preceding AP upstroke in failing human LV mycardium. Interestingly, such calcium-mediated PVCs were localized at the epicardium and midmyocardium, where (CaD–APD) is prolonged. In heart failure, given NCX activity has not been further augmented by \(\beta_2\)-stimulation because of receptor uncoupling from G\(_i\), the substrate (CaD–APD) could play a crucial role in DAD initiation (Figure 8A and 8B). In contrast, \(\beta_2\)-stimulation did not provoke DADs in failing hearts.

**Purkinje Fibers Are Responsible for the Majority of Automaticity During \(\beta_2\)-Stimulation in Heart Failure**

Low-frequency PVCs in normal hearts are considered benign, but frequent PVCs in failing hearts are associated with LV dysfunction.\(^28\) In our study, the majority of PVCs originated from the connexin 40–positive subendocardium. This suggests that PVCs evoked by \(\beta_1\) and \(\beta_2\)-AR stimulation arise in the Purkinje fibers in heart failure. Furthermore, it indicates that both subtypes may preserve the functional state of Purkinje fibers, different from that of working myocytes. Additionally, \(\beta_2\)-stimulation also evokes DAD-induced triggered activity in the subepicardium and midmyocardium. Therefore, only \(\beta_2\)-stimulation, not \(\beta_1\)-stimulation, leads to increased arrhythmia vulnerability by facilitating arrhythmogenic triggers and substrates. This specificity suggests that nonselective or \(\beta_2\)-selective blockers may be more effective in treating and preventing arrhythmogenesis in heart failure than \(\beta_1\)-selective blockers.

**Limitations**

A possible limitation of our study is our use of specific \(\beta_1\)- and \(\beta_2\)-agonists instead of a commonly used nonspecific agonist combined with isoform-specific antagonists. We selected isoform-specific agonists procaterol (1 \(\mu\)mol/L)\(^12\) and xamoterol (1 \(\mu\)mol/L)\(^13\) because of their established specificity in the settings of heart failure. Furthermore, it was not possible to do a dose response investigation because of the limited number of human hearts and limited time available for experiments. We decided not to use an alternative approach based on isoproterenol combined with \(\beta_1\) and \(\beta_2\)-blockers because we wanted to investigate the 2 types of receptors in the same heart, with access to a limited number of hearts. We acknowledge that the lack of dose response data limits our ability to compare the relative contributions of \(\beta_1\) and \(\beta_2\) to excitation–contraction coupling. Therefore, our comparisons were only between donor and failing hearts. Furthermore, we confirmed the selectivity of \(\beta_1\)-agonist xamoterol, which had dramatic stimulation effects.
in donor but not in failing hearts, consistent with previous findings of desensitization of β1-AR in heart failure.9

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Figure 8. Illustrated schematic summaries of β-adrenergic receptor (AR) signaling remodeling and electrophysiological effects in heart failure. A, Schematic highlighting the switch of β2-AR coupling from inhibitor G-protein (Gi) to stimulatory G-protein (Gs), which alters downstream regulation from a negative to a positive feedback control system. B, Summary graphic of left ventricular (LV) electrophysiological responses to β2- and β1-stimulation in heart failure. APD indicates action potential duration; ΔAPD, APD reduction; CAD, [Ca2+]i transients duration; ΔCaD, CaD shortening; DAD, delayed afterdepolarization; NCX, Na–Ca exchanger; PKA, protein kinase A; and SR, sarcoplasmic reticulum.

Disclosures

None.

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Detailed Material and Methods:

Complete and detailed material and methods for preparation, optical mapping, Western blot, quantitative-polymerase chain reaction (q-PCR) and data analysis are provided below.

Clinical information for human hearts

Procurement and research of human hearts was approved by the Institutional Review Board of Washington University School of Medicine. Non-ischemic failing human hearts (F, n=8) were obtained during transplantation at the Barnes-Jewish Hospital of Washington University School of Medicine. Donor hearts (D, n=8) were procured from the Mid-America Transplant Services (St. Louis, MO), as they were rejected for transplantation for reasons such as age. Ejection fraction values were obtained from echocardiography, which was conducted at variable times. All donor hearts were characterized as hearts without history of heart failure. The clinical data of patients are shown in Supplemental Table1.

Explanted hearts were cardioplegically arrested in the operating room following cross clamping of the aorta. Immediately after the heart was received, 100 ml 0°C cardioplegic solution (in mmol/L: NaCl 110, CaCl₂ 1.2, KCl 16, MgCl₂ 16, NaHCO₃ 10; pH=7.3±0.05) was perfused through right and left coronary
arteries respectively to remove the blood and to protect myocardium from ischemia. The heart was then bathed in 0-4°C cardioplegic solution for transportation, dissection and cannulation.

**Left ventricular wedge tissue preparation**

The arterially perfused left ventricle wedge preparations were made as previously described.\(^1\) \(^2\) Briefly, preparations were dissected approximately from the same area of the hearts to minimize tissue differences. The size of the preparation is approximate 8 cm in length, 3 cm in width, and 3 cm in thickness. Before dissection, the quality of perfusion was verified by injection of Methylene Blue dye (Sigma, St. Louis, MO). A segment of left ventricular wedge along a lateral marginal artery was isolated based on the blue color in areas supplied by dye solution. Each wedge contained a section of coronary artery along its length. The wedge was cannulated with a custom flexible plastic cannula. The quality of the perfusion was again tested by the Methylene blue dye, and poorly perfused surfaces were trimmed. Major arterial leaks in the wedges were ligated with silk suture to maintain pressure. (Supplemental Figure 1).

**Simultaneous optical mapping of action potential (AP) and calcium transient (CaT)**
The isolated tissues were mounted in a warm chamber with the dissection exposed transmural surface up, facing the optical apparatus. The preparation was both superfused and coronary perfused with oxygenated Tyrode solution (in mmol/L: 128.2 NaCl, 4.7 KCl, 1.19 NaH$_2$PO$_4$, 1.05 MgCl$_2$, 1.3 CaCl$_2$, 20.0 NaHCO$_3$, and 11.1 glucose, and gassed with 95% O$_2$-5% CO$_2$; pH=7.35±0.05). We maintained a solution temperature of 37 °C and an arterial pressure of 60-70 mmHg. Continuous monitoring of pH and temperature was conducted during the experiment. The preparation was fully immersed in the perfusion efflux, which assured proper superfusion.

After 30 minutes of washout to remove excessive fat and blood residues, gradual warming after cold cardioplegia to 37 °C, tissue recovery, and stabilization, Blebbistatin (10 to 20μmol/L; Tocris Bioscience, Ellisville, MO) was perfused into the preparation to suppress motion artifacts. After tissue was completely stabilized, RH237 and Rhod-2 AM were applied as probes for AP and CaT, respectively.

The dual optical mapping apparatus consisted of two MiCAM Ultima-L CMOS cameras (SciMedia, Costa Mesa, CA) that have high spatial (100×100 pixels, with the field of view of 3cm×3cm) and temporal (1,000 frames/sec) resolution. A band-pass filter (590±15 nm, Thorlabs, Newton, NJ) was placed in front of the calcium imaging camera; a long-pass filter (>700 nm, Thorlabs, Newton, NJ) was positioned in front of the voltage imaging camera. The cameras were arranged perpendicular to one another by a holder, which contained a
dichroic mirror (635 nm cutoff, Omega Optical, Brattleboro, VT). Immediately below the dual camera holder were a group of lenses (100mm and 50 mm focal length, f 0.95; Navitar), which helped to focus the emission light coming from the heart onto the CMOS sensors with adjustable field of view. The excitation light was generated by a halogen lamp (Newport Oriel Instruments, Stratford, CT; SciMedia, Costa Mesa, CA) and was passed through a heat filter, shutter, and band-pass filter (520±45 nm). A flexible light guide directed the band-pass filtered light onto the preparation, and a shutter was used to ensure that the preparation was exposed to light only during image acquisition to avoid photobleaching of the dyes.

**Experimental protocol**

Specific agonists for β<sub>2</sub>-AR and β<sub>1</sub>-AR procaterol (1µmol/L)<sup>3,4</sup> and xamoterol (1µmol/L)<sup>5,6</sup>, respectively, and non-specific β-AR agonist isoproterenol (ISO, 100nmol/L) (Tocris Bioscience, Ellisville, MO) were perfused into the preparation. Altering the order of β<sub>1</sub>-agonist application did not change its effect on APD reduction (**Supplemental Results**). The agonists were applied at saturating concentrations according to previous cardiac studies<sup>3-6</sup> so that the maximum effective activation of the receptors could be achieved. Conclusions are carefully driven based on comparisons between donor and failing hearts under the effects of different β-AR selective agonists, respectively.
Complete washout evaluated by the recovery of action potential duration (APD) was applied between Xamoterol and Procaterol. Extra blebbistatin and dye were loaded as needed. Programmed electrical stimulation (PES) S1S1 protocol was applied with the pacing electrode placed at subendocardium at twice the diastolic pacing threshold. Pacing cycle length (CL) ranged from 4000 to 400 ms as shown in Supplemental Figure 2. Representative mapping recordings were collected during each condition. Real time monophasic action potential (MAP) and pseudo ECG were continuously monitored in parallel with and in validation of optical mapping. 1 ml of Lugol’s solution was painted to the endocardium surface after enhanced automaticity was observed, followed by quick superfusion of Tyrode solution.

Data analysis

Transmural surface was subdivided into areas of interest: subepicardium (epi), midmyocardium (mid) and subendocardium (endo), as defined in Supplementary Figure 1. Mapping signal was low-pass filtered at 256 Hz. AP duration (APD) and CaT duration (CaD) were measured from time point of upstrokes (dV/dt_{max} and dCa/dt_{max}) to 80% repolarization and Ca relaxation. The difference between CaD and APD, (CaD-APD) was also calculated. Conduction velocity (CV) was measured and plotted as a function of direction with transverse direction defined as zero degree (Figure 1A). Action potential-Calcium delay map (AP-CaT Delay map) shows the time difference between AP activation and Ca
release \left(\frac{d\text{CaT}}{dt_{\text{max}}} - \frac{dV}{dt_{\text{max}}}\right)\) with positive value indicating activation of AP precedes calcium release (CaT). CaT rise time was quantified to estimate the Ca release phase, which was defined as the time interval between 10% and 90% CaT upstroke value. \(T_{50}\) and Ca decay constant were calculated to evaluate the Ca extrusion fast component and slow component respectively. \(T_{50}\) measures the time period from time point of CaT reaching peak value to 50% relaxation. Ca decay constant was investigated by fitting CaT signal from 50% to 100% relaxation with exponential curve \(a\exp(-1/b^*x)+c\), where \(b\) was defined as decay constant.

**Western blot study**

Additional tissue samples from human LVs were flash frozen in liquid nitrogen, pulverized, and lysed. Proteins were separated by SDS-PAGE, followed by transfer onto nitrocellulose membranes. Incubations with primary antibodies (\(\beta_2\)-AR antibody, sc-569; p-\(\beta_2\)-AR antibody (Ser345/Ser346), sc-16718; Santa Cruz Biotechnology, Santa Cruz, CA) were performed overnight at 4°C and were carried out in 5% BSA. Incubations with secondary antibodies were performed at room temperature for 2 h. After exposure, membranes were washed and stripped using Restore PLUS Western Blot Stripping Buffer (Thermo Scientific). Membranes were reprobed using an antibody against GAPDH (anti-GAPDH, Sigma, St. Louis, MO) as a loading control. Images were processed using Multi Gauge software (Fujifilm).
**Quantitative-polymerase chain reaction**

Additional tissue samples from human LVs were preserved in RNAlater (Sigma, St. Louis, MO) before RNA extraction. Total RNA extracted was processed using RNeasy Fibrous Tissue Mini Kit and a High Capacity cDNA Reverse Transcription Kit (QIAGEN, Manchester, UK) according to the manufacture's instructions. mRNA quantification for the different targets was assessed by quantitative PCR (q-PCR) using TaqMan gene expression assays by StepOne instrument (Applied Biosystems, Grand Island, NY). The thermal profile for the q-PCR reactions followed manufacturer's instruction. Data analysis for q-PCR data used efficiency-corrected $\Delta C_t$ method to determine the relative amounts of the gene of interested that were normalized to an endogenous reference RNA of GAPDH.

**Immunofluorescence staining and imaging**

Formalin fixed sections (5 $\mu$m thinkness) were prepared from the human heart tissue right after the optical mapping experiments by the HOPE center in Washington University in St.Louis. Immunofluorescent staining of DAPI and Cx40 were applied using TSA, Plus Tetramethylrhodamine (TMR) kit (PerkinElmer, Waltham, MA). Briefly, slides were de-paraffinized via three xylene washes and rehydrated with ethanol and DI water. Slides were then put into plastic container with 10mmol/L citrate buffer and cooked in a high pressure cooker for 15 minutes. After slides were cooked and cooled they were placed into Sequenza
chamber and washed with 0.1% PBST. Slides were then incubated in 300uL of 3% Hydrogen peroxidase and washed with PBST. 300uL of 3% BSA was used to block non-specific proteins for 60 minutes at room temperature and 300uL of 1:500 dilution (PBST) of anti-rabbit Cx40 antibody (Alpha Diagnostic International, San Antonio, TX) was added. Slides were incubated overnight in Sequenza chamber at 4°C. Slides were then washed with PBST after overnight incubation and 250uL of secondary antibody was added and incubated again for 60 minutes at room temperature. After another wash, 1:50 (PBST) TSA fluorescence was added and incubated for 4 minuets at room temperature and washed. DAPI (NUCblue 1:500, Vector Laboratories, Burlingame, CA) was also added for 5 minutes and washed. Finally the slides were taken out of Sequenza chamber and covered with Vector mounting medium. Mosaic imaging were taken using Nano-Zoomer (Olympus).

**Results**

*Altering the order of β₁-agonist application did not change its effect on APD reduction*

For two failing hearts, which were not included within the failing group, we applied β₁ agonist directly after baseline rather than after β₂ stimulation and washout. APD reduction from β₁ stimulation of these two hearts are 15 and 9, 4 and 11, 10 and 9 ms at epi, mid and endo respectively. This result did not differ in terms of APD reduction from β₁ stimulation that was applied after β₂ stimulation and washout. After washing with 3L of Tyrode solution, the APD did
not return to baseline levels thus indicating that $\beta_1$ agonist could not be efficiently washed out within the experimental time course. Therefore, we designed our protocol to apply $\beta_1$ agonist after $\beta_2$ stimulation and washout as described in the methods.

**No significant difference of CaT rise time was found between Failing and Donor hearts**

To assess the remodeling in CaT morphology during Ca release and extrusion, we quantified CaT rise time, and two phases of extrusion – fast $T_{50}$ and slow Ca decay constant. No significant difference of CaT rise time was found between F and D hearts under all the conditions suggesting the change of CaT morphology was mainly due to the remodeling of Ca extrusion (Supplemental Figure 5).

**q-PCR analysis of selected $\beta$-adrenergic signaling target proteins**

q-PCR was performed to further validate our hypothesis. $\beta_1$ adrenergic receptor mRNA expression was down regulated in failing hearts, while $\beta_2$ receptor expression did not change. NCX expression was significantly up regulated in failing hearts, while SERCA2a and ryanodine receptor expression did not change. Connexin40 also showed no change in expression level, however both $G_i$ and $G_s$ were significantly increased in failing hearts (Supplemental Figure 6).
Supplemental Figures and Figure legends

Supplemental Table 1

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<th>Gender</th>
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<th>arrhythmia history</th>
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<td>NICM</td>
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<td>8</td>
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<td>NICM</td>
<td>&lt;20%</td>
<td>AFIB &amp; VTACH</td>
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Supplemental Table 1. Clinical information including gender, age, diagnosis, ejection fraction and arrhythmia history of the patients were provided. (NICM: non-ischemic cardiomyopathy; AFIB: atrial fibrillation; Aflutter: atrial flutter; VTACH: ventricular tachycardia)
Supplemental Table 2

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<th>Condition</th>
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<td>320</td>
<td>307</td>
<td>281</td>
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<tr>
<td></td>
<td>mid</td>
<td>448</td>
<td>411</td>
<td>333</td>
<td>318</td>
<td>291</td>
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<td></td>
<td>endo</td>
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<td>380</td>
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<td>300</td>
<td>296</td>
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<td>epi</td>
<td>357</td>
<td>315</td>
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<td>237</td>
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<tr>
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<td>endo</td>
<td>398</td>
<td>353</td>
<td>287</td>
<td>277</td>
<td>256</td>
<td>206</td>
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</table>

| **Failing Heart** |       |              |      |      |      |     |     |          |
| Baseline          | epi   | 534          | 504  | 447  | 384  | 338 | 261 |          |
|                   | mid   | 559          | 535  | 464  | 407  | 356 | 272 |          |
|                   | endo  | 580          | 551  | 475  | 433  | 372 | 281 |          |
| **β_2**           | epi   | 497          | 460  | 348  | 341  | 305 | 235 |          |
|                   | mid   | 521          | 485  | 382  | 357  | 322 | 254 |          |
|                   | endo  | 557          | 501  | 417  | 378  | 339 | 261 |          |
| **β_1**           | epi   | 518          | 497  | 432  | 395  | 328 | 239 |          |
|                   | mid   | 544          | 527  | 446  | 412  | 349 | 261 |          |
|                   | endo  | 570          | 544  | 463  | 437  | 369 | 273 |          |
| **β_1+β_2**       | epi   | 450          | 402  | 357  | 311  | 275 | 235 |          |
|                   | mid   | 457          | 445  | 387  | 346  | 305 | 248 |          |
|                   | endo  | 476          | 468  | 410  | 369  | 317 | 262 |          |

Supplemental Table 2. Detailed average APD_{80} value under different pacing cycle length is provided for epicardium, midmyocardium and endocardium under all conditions.
Supplemental Figure 1. Representative photos of human heart and left ventricular wedge preparation. Tissue was selected along left marginal artery as boxed in green dashed line in the left photo. Field of view and definition of different transmural layers are depicted in the right photo.
Supplemental Figure 2

Supplemental Figure 2. Detailed timeline of the experimental protocol.

- MTS BJ Hospital
- Delivery: 7 min
- Lab
- Procedure: 1 hr
- Experimental setup
- Cold Cardioplegia perfusion
- Tissue collection
- Experimental preparation
- BB and dye loading
- Experimental Protocol
- Clean up

- β2 agonist
- Washout
- β1 agonist
- ISO

Control

Restitution pacing

Pace at: 4000 ms, 2000 ms, 1500 ms, 1000 ms, 800 ms, 600 ms, 500 ms, 400 ms, 350 ms, 300 ms, 280 ms, ...... until 1:1 capture lost each pacing rate maintains 4 min

- β2 agonist: β2 adrenergic receptor agonist Procaterol 1 μM
- β1 agonist: β1 adrenergic receptor agonist Xamoterol 1 μM
- ISO: β1 and β2 adrenergic receptor agonist 100 nM
Supplemental Figure 3

Supplemental Figure 3. Conduction velocity change in response to β₂, β₁ and non-selective β agonists. β₂ agonist dramatically increased CVL but only mildly affected CVT in both groups of hearts; β₁ agonist
Supplemental Figure 4. CaD values at the baseline condition and in presence of β₂, β₁ and non-selective β-agonists from all ventricular layers.
Supplemental Figure 5. CaT rise time was calculated to evaluate the upstroke speed for CaT. No significant difference in rise time was observed between donor and failing hearts under all conditions.
Supplemental Figure 6. mRNA expression of selected β-adrenergic signaling target proteins was investigated by q-PCR. mRNA of β₁ expression is dramatically decreased in failing hearts. NCX and Gᵢ expression increased in failing hearts.
Supplemental Figure 7. Western blot of $\beta_2$ expression normalized to GAPDH.

The expression showed no significant difference between donor and failing hearts in either endocardial or epicardial layers.
References


