Spontaneous Ventricular Fibrillation in Right Ventricular Failure Secondary to Chronic Pulmonary Hypertension

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Background—Right ventricular failure (RVF) in pulmonary hypertension (PH) is associated with increased incidence of sudden death by a poorly explored mechanism. We test the hypothesis that PH promotes spontaneous ventricular fibrillation (VF) during a critical post-PH onset period characterized by a sudden increase in mortality.

Methods and Results—Rats received either a single subcutaneous dose of monocrotaline (MCT, 60 mg/kg) to induce PH-associated RVF (PH, n=24) or saline (control, n=17). Activation pattern of the RV-epicardial surface was mapped using voltage-sensitive dye in isolated Langendorff-perfused hearts along with single glass-microelectrode and ECG-recordings. MCT-injected rats developed severe PH by day 21 and progressed to RVF by approximately day 30. Rats manifested increased mortality, and ≈30% rats died suddenly and precipitously during 23–32 days after MCT. This fatal period was associated with the initiation of spontaneous VF by a focal mechanism in the RV, which was subsequently maintained by both focal and incomplete reentrant wave fronts. Microelectrode recordings from the RV-epicardium at the onset of focal activity showed early afterdepolarization-mediated triggered activity that led to VF. The onset of the RV cellular triggered beats preceded left ventricular depolarizations by 23±8 ms. The RV but not the left ventricular cardiomyocytes isolated during this fatal period manifested significant action potential duration prolongation, dispersion, and an increased susceptibility to depolarization-induced repetitive activity. No spontaneous VF was observed in any of the control hearts. RVF was associated with significantly reduced RV ejection fraction (P<0.001), RV hypertrophy (P<0.001), and RV fibrosis (P<0.01). The hemodynamic function of the LV and its structure were preserved.

Conclusions—PH-induced RVF is associated with a distinct phase of increased mortality characterized by spontaneous VF arising from the RV by an early afterdepolarization-mediated triggered activity. (Circ Arrhythm Electrophysiol. 2012;5:181-190.)

Key Words: pulmonary hypertension ■ right ventricular failure ■ early afterdepolarization ■ optical mapping ■ ventricular fibrillation

Pulmonary hypertension (PH) is a lethal syndrome caused by arteriolar obstruction resulting from excessive proliferation of pulmonary artery smooth muscle and endothelial cells, endothelial dysfunction, inflammation, and excessive vasoconstriction.1,2 Long-standing pressure overload in PH leads to right ventricular (RV) hypertrophy and subsequently diastolic and systolic RV failure (RVF).3,4 In fact, RVF is the most common cause of death in PH patients (≈30–50% of deaths). Sudden death has also been reported to account for ≈17–28% of deaths in these patients.5,6 Whereas pulmonary vascular disease has been the primary pathological focus, less attention has been paid to the potential of direct lethal arrhythmic consequences of the RV as a cause of sudden death in PH. For example, the electric7 and structural8 remodeling of the RV associated with PH could potentially provide both substrate and trigger for the initiation of spontaneous ventricular fibrillation (VF), a major cause of sudden cardiac death.9

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Ample experimental studies in rats have shown that a single injection of the toxic alkaloid monocrotaline (MCT) successfully recapitulates the major pulmonary arterial and RV pathological features of PH in humans, including the sudden and precipitous rise in mortality10. Furthermore, changes in ventricular gradient and QT interval were also seen both in PH patients11,12 and in animal models of PH.3,13,14 MCT-induced PH is found to also prolong the RV
action potential duration\textsuperscript{3,7,15} in association with downregulation of K\textsuperscript{+} channels.\textsuperscript{3, 7,14} However, to our knowledge, a combined systematic intact heart and cellular electrophysiological, hemodynamic, and histobiochemical study of the RV and the left ventricle (LV) and their respective roles in the initiation of spontaneous VF in chronic PH is still lacking. The purpose of this study was to test the hypothesis that chronic PH promoted spontaneous VF during a critical post-PH period is associated with major structural and electrophysiological remodeling of the RV but not the LV, providing both the substrate and the trigger for spontaneous VF.

Methods

Animals, Treatment, and Mortality Criteria

Chronic PH-associated RVF was induced in male Sprague-Dawley rats (3–4 months, 350–400 g) by a single s.c. injection of 60 mg/kg MCT (n=24) and compared with saline-treated control rats (CTRL, n=17). Details of the treatment and criteria for mortality are given in the online-only Data Supplement.

Cardiac and Pulmonary Hemodynamics

Serial B-Mode, M-Mode, and pulmonary pulsed wave Doppler echocardiography were performed using a VisualSonics Vevo 770 (VisualSonics, Ontario, Canada) equipped with a 30-MHz linear transducer to accurately monitor cardiopulmonary hemodynamics, as described in detail in the online-only Data Supplement. RV and LV pressures were measured by direct cardiac catheterization at the end of the 4th week before euthanasia.

Whole-Heart Isolated, Perfused Langendorff Studies and Optical Mapping

After excision of the heart, the ascending aorta was cannulated for retrograde perfusion with warm (36.5±0.5°C) oxygenated Tyrode solution and mounted in a tissue bath for optical mapping of RV activation patterns, as we previously described.\textsuperscript{16} The hearts were stained with the voltage-sensitive dye, RH237 (Invitrogen Molecular Probes, Carlsbad, CA) for fluorescent optical mapping of the RV epicardial surface. Cytochalasin D (5 μmol/L) was added to the perfusate to inhibit motion. Single-cell action potentials were recorded with glass microelectrodes from selected RV epicardial surface areas that optical mapping showed focal activity in order to determine the cellular mechanisms of the focal activity. An epicardial wave front was considered to be focal when it arose from within the RV mapped region (ie, did not propagate into the mapped region from the outside) and was surrounded by recovered tissue.\textsuperscript{16} A total of 9 hearts were studied (n=5 for CTRL and n=4 for PH).

Isolated Cardiomyocyte Studies and Immunocytochemistry Imaging

At the end of the 4th week, the hearts were dissociated with collagenase. RV and LV cardiomyocytes were isolated as described before\textsuperscript{8} and in the online-only Data Supplement. A total of 10 hearts were used (n=5/group). Freshly isolated myocytes were fixed and stained with antibodies. Images were acquired with a high-resolution confocal microscope.

Figure 1. Cardiac hemodynamics and mortality in rats with pulmonary hypertension (PH). A shows echocardiographic images of B-mode (left panel), M-mode of heart (middle panel), and pulsed wave Doppler of pulmonary artery (PA) flow (right panel), where EDD is right ventricular (RV) end-diastolic diameter. Arrows in the pulsed wave Doppler signals show midsystolic notch in the pulmonary artery flow in PH (n=7 for control [CTRL] and n=6 for PH). B shows hematoxylin and eosin staining of heart cross sections of CTRL and PH (n=7 for CTRL and n=6 for PH). C shows combined time course of mortality (including sudden unexpected deaths, black squares) and RV ejection fraction (RVEF) (black circles) in the PH group (n=24). LV indicates left ventricle; IVS, interventricular septum; and MCT, monocrotaline.
Whole-Cell Patch-Clamp Experiments
Membrane potentials of RV cardiomyocytes (n=5/group) were measured with the patch-clamp technique in the whole-cell current-clamp configuration. The details of patch-clamp experiments are described in the online-only Data Supplement.

Gross Histological Evaluation
The RV wall, the LV wall, and the interventricular septum (IVS) were dissected, weighed, and the weight ratio of RV/(LV+IVS) was calculated as an index of RV hypertrophy. The LV/body weight ratio was used as an index for LV hypertrophy.

Real-Time PCR and Western Blot Analysis
Standard real-time PCR and Western blot were performed. The details of procedures are described in the online-only Data Supplement.

Immunohistochemistry and Imaging
Hearts were fixed, and transversal 6- to 7-μm sections were obtained. Tissue sections were stained with immunofluorescence, standard hematoxylin and eosin, and Masson trichrome stain (see online-only Data Supplement for details).

Statistical Analysis
Means were compared between the control and PH groups with the use of t tests, as all outcomes were well modeled by gaussian (normal) distribution. A 2-sided probability value of <0.05 was considered statistically significant. Means and standard errors of the mean (SEMs) are reported.

Results
Effects of Chronic PH on Mortality, Cardiac Hemodynamics, and Cardiac Structure
Severe PH was evident ~4 weeks after MCT injection by the presence of midsystolic notching on pulmonary artery flow profile in pulsed wave Doppler echocardiography (Figure 1A). Direct RV catheterization confirmed the severity of PH as the RV peak systolic pressure (RVPSP) was 2.5-fold higher in the PH group than in the controls (Table). LV pressures remained unchanged ~4 weeks after MCT treat-
MCT-induced PH selectively depressed the function of the RV as demonstrated by the decrease of the RVEF from 72% at day 14 to 38% at day 21 (Figure 1C). Thereafter and up to the time of the sacrifice, the RVEF of the MCT-treated rats remained in the range of 30–35%. In contrast to RV, the LV function was fully preserved with no change in the LVEF for the entire 30 days after MCT period (Table). Sudden unexpected deaths in the PH group occurred from day 23 onward (30%), and all rats died by day 32 after MCT treatment (Figure 1C). We defined the period between days 23–32 as the “sudden death period.” It is of interest to note that the precipitous drop in the RVEF from days 14–21 preceded by about 10 days the precipitous rise in the incidence of sudden deaths in the MCT-treated rats. The lack of temporal correlation between the RV hemodynamic worsening and the emergence of spontaneous VF suggests that it is the electric failure rather than the hemodynamic deterioration as the primary inciting factor for the VF (Figure 1C). MCT-induced PH caused major structural changes in the RV but not in LV. The RV changes included hypertrophy as reflected by the increased ratio of RV/(LV+IVS) weights and an increase in the RV cardiomyocyte size (Table and Figure 2A and 2B). A significant increase in RV fibrosis both in the epicardium (17±2% versus 1.5±0.4% in CTRL) and in the endocardium (18±3% versus 1.4±0.3% in CTRL; Figure 2C) was evident in the MCT-induced PH group. In addition, the expression levels of the proapoptotic cleaved caspase-3 protein were significantly increased in the RV, reflecting the presence of apoptosis/necrosis in the failing RV (Figure 2D). In contrast to the RV, the structure of the LV was preserved, as there were no signs of hypertrophy, fibrosis, or apoptosis.

Spontaneous Initiation of VF in Chronic PH Rat Hearts

To gain insight into the cellular mechanism of spontaneous VF initiation, we continuously monitored the ECG, LV, and LA bipolar electrograms and single-cell action potentials recorded with microelectrode from the base of the RV epicardium of the isolated RV failed hearts during the sudden death period (ie, days 23–32 after MCT). Sudden unexpected deaths in the PH group occurred from day 23 onward (30%), and all rats died by day 32 after MCT treatment (Figure 1C). We defined the period between days 23–32 as the “sudden death period.” It is of interest to note that the precipitous drop in the RVEF from days 14–21 preceded by about 10 days the precipitous rise in the incidence of sudden deaths in the MCT-treated rats. The lack of temporal correlation between the RV hemodynamic worsening and the emergence of spontaneous VF suggests that it is the electric failure rather than the hemodynamic deterioration as the primary inciting factor for the VF (Figure 1C). MCT-induced PH caused major structural changes in the RV but not in LV. The RV changes included hypertrophy as reflected by the increased ratio of RV/(LV+IVS) weights and an increase in the RV cardiomyocyte size (Table and Figure 2A and 2B). A significant increase in RV fibrosis both in the epicardium (17±2% versus 1.5±0.4% in CTRL) and in the endocardium (18±3% versus 1.4±0.3% in CTRL; Figure 2C) was evident in the MCT-induced PH group. In addition, the expression levels of the proapoptotic cleaved caspase-3 protein were significantly increased in the RV, reflecting the presence of apoptosis/necrosis in the failing RV (Figure 2D). In contrast to the RV, the structure of the LV was preserved, as there were no signs of hypertrophy, fibrosis, or apoptosis.

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trogram and the QRS of the pseudo-ECG by 14±6 ms and 28±8 ms, respectively (6 episodes, 4 hearts) (Figure 3). This indicates that the triggered activity arising from the RV drives the LV. The VF s were terminated by electric shocks, and several minutes later, another episode of spontaneous VF reemerged. In control saline-treated rat hearts, no VF emerged for up to 3 hours of perfusion with normal Tyrode solution (n=5). To determine whether the RV epicardial surface of the MCT-treated hearts manifests conduction block that might result from an anatomic obstacle in the failing and structurally remodeled fibrotic RV, we optically mapped the RV epicardial surface using voltage-sensitive dye RH-237. As shown in Figure 4A, no conduction block developed over the epicardial surface of the failed RV during either pacing or during normal sinus rhythm indicating the absence of anatomic obstacles over the epicardial surface of the failed RV. To determine the mechanism(s) by which the VF in the failing RV is maintained once initiated by an EAD-mediated triggered activity; we optically mapped the RV epicardial surface in 3 MCT-treated hearts 10 seconds after the onset of VF. Both focal and incomplete reentrant wave fronts were observed during all 6 episodes of VF in all 4 hearts (Figure 5 and online-only Data Supplement Figure 2). As shown in Figure 5, the activation map during VF showed 2 competing foci (labeled 1 and 2 in Figure 5B). Although both foci had a frequency of 14 Hz, the focus originating from site 1 activated the surrounding RV myocardium with a 2:1 pattern, causing the surrounding portion of the RV to be activated at a frequency of 7 Hz (Figure 5C). The optical action potentials recorded from the 2 foci and the RV surrounding site with a 2:1 block are shown in Figure 5D. Focal activity was defined as an island of depolarized tissue on the epicardial surface surrounded by recovered tissue.

Mechanisms of Increased Susceptibility of PH Hearts to EADs and Triggered Activity

Theoretical and experimental studies have shown that reduced repolarization reserve, increased myocardial fibrosis, and elevated cytosolic Ca levels promote EADs and triggered activity that could lead to VF.16–21 Cardiomyocytes isolated from failing RV, unlike cardiomyocytes isolated from the LV, had their action potential duration (APD)90 (90% repolarization) prolonged from 33±6 ms (control) to 57±8 ms in the MCT-treated hearts (P<0.001) (Figure 6A), suggesting reduced repolarization reserve, a major characteristic of failing cardiomyocytes.22 Furthermore, cells isolated from the

Figure 4. Optical snapshots, isochronal maps, and optical action potentials (OAPs) recorded from the right ventricular (RV) epicardium in a heart 29 days after monocrotaline injection. A (left) are snapshots during RV pacing from the base at a cycle length of 200 ms and during sinus rhythm (right, 3 snapshots) showing total RV epicardial activation within 10 ms. Asterisk at the base of the RV is the pacing site (downward white arrow indicates the direction of propagation). Notice the absence of conduction block during pacing and during sinus rhythm. Time zero is chosen arbitrarily for both pacing and sinus rhythm snapshots. B is a schematic drawing of OAP with blue denoting repolarization and red, depolarization. C, Isochronal maps during pacing (left) and during sinus rhythm (right) of the corresponding snap shots shown in A. Arrows in C indicate the direction of wave front propagation. D shows simultaneous 8 OAPs recorded from equally spaced sites (1–8) identified in C (black arrow). Downward pointing red arrow indicates the direction of wave front propagation during RV pacing from the base and upward pointing red arrow indicates sinus beats. The lower 4 panels in D are simultaneous recordings of pseudo-ECG, left ventricular and atrial bipolar electrograms (LV Beg and LA Beg, respectively), and pacing stimulus artifact (bottom tracing). Six paced beats at a cycle length of 200 ms are followed by 5 beats of sinus origin with a cycle length of ~310 ms.
RV of MCT-treated hearts manifested significantly greater dispersion in APD90 (maximum APD minus minimum APD) compared with control (30 ± 1.8 ms versus 20 ± 1.5 ms, 16 cells in 5 hearts; \( P < 0.01 \)). Because the potential of depolarizing influences exerted by fibroblasts in hearts with increased fibrosis could promote EADs and triggered activity,\(^a\)\(^b\)\(^c\) we tested the influence of long-duration (1.6 seconds) depolarizing current pulses on depolarization-induced triggered activity in isolated control and failing RV cardiomyocytes. Unlike the LV cardiomyocytes, RV cardiomyocytes isolated from MCT-treated hearts manifested triggered activity with higher frequency (Figure 6B) and higher amplitudes than control RV cardiomyocytes in response to a given depolarizing current pulse (4.2 ± 0.37 versus 2.5 ± 0.28 action potentials per 1.6-second pulse, \( P < 0.01 \), and 65.2 ± 4.7 mV versus 45 ± 3.5 mV measured at the end of the 1.6-second period, \( P < 0.05 \), respectively). Electrophysiological changes seen in PH myocytes could not be attributed to direct effects of MCT because bath application of MCT had no effect on action potential configuration in isolated RV and LV myocytes\(^7\) (online-only Data Supplement Figure 3).

In summary, our findings indicate that cardiomyocytes from the PH group with reduced repolarization reserve manifest greater susceptibility to generate triggered activity that could lead to VT and VF. To determine the possible molecular mechanisms associated with reduced repolarization reserve, we examined the expression of sarcoplasmic reticulum calcium-sensitive ATPase (SERCA2a) as well as the molecular correlates of the major repolarizing currents in rodents, for example, \( I_{\text{K\text{-slow}}} \), \( K_{\text{v1.5}} \), and \( K_{\text{v2.1}} \) in the RV and LV of PH rats during the sudden cardiac death period (ie, 23–32 post-MCT treatment days). PH caused a selective and considerable reduction (by 94%) in the level of SERCA2a protein in the RV of PH group (0.06 ± 0.01) versus control (1.00 ± 0.26, \( P < 0.05 \)) (online-only Data Supplement Figure 1) without affecting SERCA2a level in the LV. Real-time PCR revealed that \( K_{\text{v1.5}} \) transcripts but not \( K_{\text{v2.1}} \) were downregulated by 50% in the RV of PH hearts (Figure 7A). Consistent with the lower \( K_{\text{v1.5}} \) transcript levels in the PH group, \( K_{\text{v1.5}} \) protein levels in the RV were also downregulated by 88% (from 1.00 ± 0.26 to 0.12 ± 0.03, \( P < 0.05 \)) but not in the LV (Figure 7B). \( K_{\text{v1.5}} \) immunoreactivity was also lower in RV cardiomyocytes in the PH group but not in the LV cardiomyocytes (Figure 7C). \( KCNE2 \), an ancillary \( K^+ \) channel subunit, has been shown to physically associate with \( K_{\text{v1.5}} \) in murine heart,\(^{23}\) and its ablation has been shown to result in 50% reduction of \( I_{\text{K\text{-slow}}} \) leading to APD prolongation.\(^{23}\) \( KCNE2 \) protein expression was ~4-fold lower in the

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**Figure 5.** Optical imaging during spontaneous ventricular fibrillation (VF) in a heart isolated 29 days after monocrotaline injection. Open stars indicate the 2 foci; white arrows indicate propagation from foci; and black arrows indicate propagation from existing waves. A shows the pseudo-ECG of the onset of the spontaneous VF. The heart was imaged about 15 seconds after onset. The pECG of the imaged period is shown in the second part of the trace, after the 10-second break. The red double arrow indicates the period shown in B. B shows snapshots of activation during 144 ms of imaged time. The red dots in the first image indicate the locations of the optical action potentials (OAPs) shown in D. See D for the color scale of the snapshots in relation to the OAPs. C shows the frequency map with 2 sample OAPs at the closed circle and square. D shows OAPs highlighting the propagation from the foci 1 (OAPs a and b) and 2 (OAPs c, d, and e) indicated in B. E shows the phase (\( \phi \)) map of the 7-Hz activity. The activity continued and arrived at the apex beyond the end of the period (ie, the foci had already fired the second time), so this late activity near the apex was unwrapped to the \( 2\pi \) complement for clarity.
Selective RV Remodeling Secondary to Chronic PH

In the present study, we show selective functional, structural, and electric remodeling only in the RV but not in the LV of the PH group, consistent with previous reports. However, relatively minor LV remodeling has also been reported in chronic PH. For example, Lamberts et al. have shown structural changes in the LV resulting in a moderately depressed LV diastolic function along with minor increase in LV fibrosis. Benoist et al. using the MCT model of PH in rats, found that the remodeled RV had a steeper slope of monophasic APD restitution curve than the LV, causing increased susceptibility to rapid pacing-induced VF in these remodeled hearts. Furthermore, these authors found that the prolongation of the APD was considerably more in the RV compared with the LV (90% versus 15%).

Mechanism of Spontaneous VF

Single-cell microelectrode recording from the RV epicardial surface near the base showed that the spontaneous VF was initiated by EAD-mediated triggered activity causing VT, which then degenerates to VF (Figures 3 and 5). Importantly, the emergence of EADs on the RV epicardial surface coincident in time with the isoelectric interval of the simultaneously recorded LV and pseudo-ECG (Figure 3A) and preceded the activation of the LV indicating that the failed RV was driving the LV during the EAD-mediated triggered activity. This finding suggests that the RV is the site of origin of the trigger for VF in rats with severe PH induced by MCT. In addition, the increased incidence of deaths occurring in the failing RV cardiomyocytes could promote reentry that may contribute to the maintenance of VF. In fact, spontaneous VF is maintained by both focal and incomplete reentrant mechanisms. It may be argued that the increased incidence of deaths may directly result from the severe RV hemodynamic deterioration associated with chronic PH. However, the differential time course of RV hemodynamic deterioration and increased mortality argues for RV electric dysfunction rather than the depressed hemodynamic function of the RV as a primary cause of death in our rat model of MCT-induced PH. Indeed, the RV function deteriorated precipitously between days 14–21 as the RVEF fell steeply from ≈72% at day 14 to ≈38% at day 21 at a time period when most of the PH rats were still alive. The highest incidence of sudden and precipitous deaths occurred between days 23–32, some 10 days after the maximum attainable decrease in RVEF (Figure 1C). These findings suggest that the mortality during the sudden death period is caused by EAD-mediated triggered activity in the RV. However, we cannot exclude the potential role of severe RV hemodynamic depression in the initiation of spontaneous VF in vivo. It is possible that the depressed RVEF may conspire with electric remodeling to promote VF in vivo. A recent report showed increased susceptibility to electrically inducible arrhythmias in rat hearts with MCT-induced PH; however, no spontaneous VF, was documented in this study.

Molecular and Structural Determinants of EAD-Mediated Triggered Activity

Whereas EADs are readily induced in isolated ventricular cardiomyocytes, EADs at the tissue level may be suppressed by source-to-sink mismatches arising from cell-to-cell coupling. That is, a small current that is sufficient to reverse repolarization and cause an EAD in an isolated cardiomyocyte will be diluted into adjacent repolarizing cardiomyocytes (unless they are also simultaneously primed for an EAD), thereby suppressing the EAD. The increased RV fibrosis observed in the MCT-treated hearts promotes partial cellular uncoupling by interstitial collagen deposition that effectively reduces that sink effect, thereby allowing susceptible cardiomyocytes in the RV to generate EADs and triggered activity causing VF.
In addition to increased fibrosis in the RV, relatively selective reduction of RV myocyte repolarization reserve could also facilitate the formation of EADs. In this respect, selective downregulation of Kv1.5 in RV but not LV, as shown here and in previous studies, may contribute to the enhanced susceptibility of the RV myocytes to generate EADs in the PH group. Furthermore, selective downregulation of KCNE2, an ancillary K+ channel subunit, and its disappearance from T-tubules in the RV but not the LV in the MCT-treated rats may contribute to RV arrhythmogenesis as inherited mutations in this gene are associated with human cardiac arrhythmogenesis. Disappearance of KCNE2 from the T-tubules during sudden cardiac death period further disrupts its association with Kv1.5 that may result in further reduction of repolarization reserve. The reduced repolarization reserve of the failing RV cardiomyocytes was associated with increased frequency and increased amplitude of depolarization-induced triggered activity, further supporting the mechanism of cellular EAD-mediated triggered activity recorded in the whole heart. Selective downregulation of SERCA2a protein in failing RV cardiomyocytes as shown here and by others is expected to slow Ca2+ ion uptake by the sarcoplasmic reticulum after each beat, causing an elevation of intracellular Ca2+ concentration. This phenomenon is known to activate the forward mode for the Na+–Ca2+ exchanger providing a net inward current that further reduces repolarization reserve and facilitates EAD formation in the failing RV cardiomyocytes. In addition to SERCA2a, Kogler et al. found that other calcium-handling proteins such as phospholamban and ryanodine receptor were also downregulated only in the RV but not in the LV of MCT-treated rats. In a recent study, Miura et al. suggested that an increase in diastolic [Ca2+]i and an increase in Ca2+ sensitivity of the sarcoplasmic reticulum Ca2+ release channel accelerate Ca2+ waves in ventricular hypertrophy, thereby causing arrhythmogenesis in the RV of MCT-induced PH.

Limitations
In epicardial surface maps, an intramural wave front breaking through the surface may have the appearance of a focal activation. The fibrotic and apoptotic/necrotic changes in the endocardium of the RV may limit breakthroughs, suggesting an intrinsic role of the epicardial cells in the genesis of triggered foci. In our previous study, we have shown that the destruction of the endocardial Purkinje network by cryoablation did not prevent epicardial muscle cells to generate EADs,
suggesting an intrinsic ability of epicardial cardiomyocytes to generate EAD-mediated triggered activity. The presence of EADs generated by epicardial cardiomyocytes of the failing RV, which occurs during the isoelectric interval of the pseudo-ECG and, more importantly, precedes LV activation, further supports this intrinsic ability of the epicardium to generate EAD-triggered activity. The presence of these right ventricular-generated EADs further suggests that the failing RV drives the LV during the EAD-triggered cardiac activity.

**Clinical Implications**
The mechanism of increased premature mortality in RV failure associated with chronic PH is still unresolved. Chronic PH is associated with increased incidence of sudden cardiac death by mechanisms that are not fully understood. We report that VF can be a potential mechanism of sudden premature death during chronic PH induced by MCT, a model of experimental PH that recapitulates the major features of experimental PH that recapitulates the major features of severe PH to determine the incidence of spontaneous VF as a potential mechanism of death in this dreadful syndrome.

Although idiopathic PAH is perceived as a progressive disease with uniformly poor outcome, the natural history of the disease is heterogeneous, with some patients dying within months and others living for decades. Despite the advances in vasodilator/vasomodulating therapies altering the course of the disease, still the most common causes of death remain RV failure and sudden death. To the extent that our findings in the MCT rat model would bear resemblance to human PH-induced RV failure, spontaneous VF may be considered a potential mechanism of death in this dreadful syndrome. However, more clinical work is needed in patients with severe PH to determine the incidence of spontaneous VF as a cause of premature death in these patients.

**Conclusions**
Chronic PH is associated with selective electrophysiological, hemodynamic, and structural remodeling of the RV but not the LV. Selective RV downregulation of (1) the molecular correlates of IK\textsubscript{Slow} expression Kv1.5 and KCNE2, (2) downregulation of SERCA2a, (3) increased RV fibrosis, (4) increased RV apoptosis and/or necrosis promote(s) EAD-induced activity of the RV, which then lead(s) to VF during a critical post-PH vulnerable period of RV failure. This mechanism, combined with severe RV hemodynamic compromise, may conspire to account for the high rate of sudden deaths in patients with chronic PH.

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None.

**References**


**CLINICAL PERSPECTIVE**

Chronic pulmonary hypertension (PH) is associated with increased incidence of sudden cardiac death by mechanisms that are not fully understood. We report that ventricular fibrillation (VF) can be a potential mechanism of sudden premature death in an experimental model of chronic PH. However, we cannot exclude the potential role of depressed contractile right ventricular (RV) function and RV ischemia that could conspire with the electrical remodeling to facilitate the emergence of early afterdepolarization–mediated ventricular tachycardia and VF. Although idiopathic PH is perceived as a progressive disease with uniformly poor outcome, the natural history of the disease is heterogeneous, with some patients dying within months and others living for decades. Despite the advances in vasodilator/vasodulating therapies altering the course of the disease, still the most common causes of death remain RV failure and sudden death. To the extent that our findings in the rat model would bear resemblance to human PH-induced RV failure, spontaneous VF may be considered a potential mechanism of death in this dreadful syndrome. However, more clinical work is needed in patients with severe PH to determine the incidence of spontaneous VF as a cause of premature death in these patients.
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SUPPLEMENTAL MATERIAL

Animals, treatment and mortality. Male Sprague-Dawley rats (350-400 g) were used. To induce PH, rats were treated with a single subcutaneous injection of MCT (60 mg/kg, Sigma, St. Louis, MO). This model has been extensively studied by many investigators and has been shown to be reproducible\textsuperscript{1,2}. MCT was dissolved in 1N HCl, the pH was adjusted to 7.4, and diluted with PBS before injection. A weight loss of more than 10% per day for 2 consecutive days and arterial oxygen saturation of less than 80% were the criteria required to sacrifice the animals, which were counted as a ‘loss’ in survival measurements. Both sudden deaths as well as the animals counted as ‘loss’ were included in the mortality plot. Sudden death was defined as unexpected death of animals without significant hemodynamic compromise, and without reaching the criteria required for sacrifice, and these sudden deaths started as early as day 23. The sudden death animals were only used for mortality plot and the hearts of these animals were not used for molecular, biochemical or electrophysiological experiments. Protocols received institutional review and committee approval. The investigation conformed to the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996).

Cardiac and pulmonary hemodynamics. B-mode, M-mode and pulmonary pulsed wave Doppler echocardiography was performed using a VisualSonics Vevo 770 (VisualSonics, Ontario, Canada) equipped with a 30-MHz linear transducer. During the course of the experiments, serial echocardiography was performed to accurately monitor the stage of the disease by measuring cardiac and pulmonary hemodynamic parameters,
as well as RV structure. Peak systolic RV pressure was calculated from pulsed wave Doppler echocardiography of pulmonary artery flow using Mahan’s regression equation: MPAP= 79 – 0.45×PAAT, in which MPAP is mean pulmonary artery pressure and PAAT is the pulmonary artery acceleration time\(^3\). The RV pressure was also measured directly by inserting a catheter (1.4F Millar SPR-671, CO, USA) connected to a pressure transducer (Power Lab, ADInstruments, CO, USA) into the RV right before sacrifice. The values of RV pressure measured by both methods were very similar. The RV ejection fraction, RV free wall thickness and RV cavity dimensions were quantified using M-mode.

**Gross histologic evaluation.** The RV wall, the left ventricular (LV) wall and the interventricular septum (IVS) were dissected and the weight ratio of the RV to LV plus IVS [RV/(LV+IVS)] was calculated as an index of RV hypertrophy. LV/body weight ratio was calculated as an index of LV hypertrophy.

**Isolation of cardiomyocytes**

The heart was quickly removed and perfused through the aorta with the following solutions: (i) Ca\(^{2+}\)-free Tyrode solution for 5 min, (ii) Ca\(^{2+}\) -free Tyrode solution containing 160 U/mL collagenase Type II (Worthington Co., Ltd, Freehold, NJ) and 0.45 U/ml protease Type XIV (Sigma) for ~15 min; and (iii) Krebs solution for 5 min. The solutions were oxygenated with 5% CO\(_2\) and 95% O\(_2\) prior to use and were maintained at 37±1°C. Freshly isolated cardiomyocytes were used for electrophysiology or immunocytochemistry.
**Immunocytochemistry and imaging.** Freshly isolated RV and LV cardiomyocytes were fixed in cold acetone for 10 min at -20°C. The cells were incubated with 10% normal goat serum (NGS) to block the background and were then stained with primary antibodies in 1% NGS and 0.1% Triton X-100 in PBS at 4°C overnight. Cells were incubated with goat anti-rabbit IgG-AlexaFluor-488 or goat anti-mouse IgG-AlexaFluor-568 secondary antibodies (Invitrogen). Images were acquired at a spatial resolution of 17391 pixels per μm with a confocal microscope (Olympus Fluoview).

**Whole-cell patch-clamp experiments**

The recordings were performed with an L/M-PC amplifier (List-Medical, Darmstadt, Germany), set at 3 KHz filtering. pClamp/Clampex8 software (Axon Instruments, Molecular Devices, Sunnyvale, CA) was used for data acquisition and off-line analysis as previously described. The bath solution contained (in mmol/L) NaCl 137, KCl 5, MgCl2 1, CaCl2 1.8, HEPES 10, and glucose 11, pH 7.4, while the pipette solution contained (in mmol/L) Na2ATP 6, KCl 115, MgCl2 1, EGTA 5 and HEPES 10, pH 7.4). Pipette resistance was 2.8±0.4 MΩ, seal resistance was 4.1±1.3 GΩ, and series resistance (Rser) was 6.6±3.1 MΩ. Whole-cell recordings after gigasealing were accepted for analysis if the membrane potential stabilized within a few minutes and if the membrane resistance measured in voltage-clamp at −60 mV (Rm(-60)) was >100 MΩ (to avoid leaky membranes). Long duration (2000 ms) depolarizing current pulses promoted triggered activity in cardiomyocytes from control rats as well as from rats with MCT-induced RV failure. Action potential duration (APD) at 90% repolarization (APD90), dispersion of repolarization (DR) as assessed by the difference between the maximal and minimal
APD, frequency and peak amplitudes of triggered activity were measured using pClamp/Clampex8 software.

**Real time PCR**

Total RNA from RV and LV was isolated using Trizol (Invitrogen, city, country) and reverse transcribed with gene specific primers using Omniscript RT kit (Qiagen, city, country). Controls were: (1) the reaction without reverse transcriptase, and (2) H₂O instead of cDNA.

**Western blot analysis**

Standard Western Blot analysis was performed using whole RV and LV lysates. Lysates were prepared by homogenizing the tissue in: 50 mmol/L Tris (pH 7.5), 1 mmol/L EDTA, 5 mmol/L MgCl₂, 150 mmol/L NaCl, 1 mmol/L DTT supplemented with phosphatase inhibitor cocktail (Roche, city, country). The samples were then centrifuged at 12,000 g for 10 min and the supernatants were collected. The protein concentrations were measured and 100 µg of protein was treated with SDS/DTT loading buffer prior to gel electrophoresis. The blots were probed with primary and secondary antibodies and visualized with the Odyssey™ Imaging System (Li-Cor). Equal loading of protein onto each lane in the gel was confirmed by analyzing the protein bands of vinculin or GAPDH. Quantification of protein levels was performed using the Metamorph software.
**Immunohistochemistry and imaging**

Heart was fixed in 4% paraformaldehyde (PFA) in 0.1 mol/L Na$_2$HPO$_4$ and 23 mmol/L NaH$_2$PO$_4$ (pH 7.4) for 4 h on ice. The tissue was then immersed in ice-cold 20% sucrose overnight to cryoprotect the tissue, mounted using OCT (Sakura Finetek, CA, USA) and transversal 6-7 μm sections were obtained with a cryostat. Tissue sections were stained with immunofluorescence, standard hematoxylin/eosin and Masson trichrome staining. The images were acquired using a light microscope (Axiovert 135, Zeiss Germany) or with a laser scanning confocal microscope (Olympus).

- **Immunofluorescence staining.** Hearts cross-sections (6 μm) were fixed in acetone for 15 minutes at –20°C. The sections were then washed with PBS+0.1% Triton X-100 three times, and incubated with 10% normal goat serum in PBS+0.1% Triton X-100 for 30 min to block the background. The sections were then incubated with primary antibodies in PBS+0.1% Triton X-100+ 1% normal goat serum at 4°C overnight. The sections were then washed with PBS+0.1% Triton X-100 three times, incubated with the appropriate secondary antibodies in PBS+0.1% Triton X-100+ 1% normal goat serum at room temperature for 1 h. For cellular hypertrophy assessment, after washing the secondary antibodies with PBS+0.1% Triton X-100 three times, the sections were incubated with wheat germ agglutinin (WGA, 1:200 dilution) in PBS+0.1% Triton X-100+ 1% normal goat serum for 1 h at room temperature. The sections were then washed with PBS three times and mounted using Prolong gold (Invitrogen) for imaging.
Quantification of cardiac fibrosis

Standard Masson trichrome staining was performed to assess cardiac fibrosis. With the use of a grid that divided the field of view into 100 squares, the number of collagenous tissue (blue stain) at the 100 intersection points in the grid was scored as 1 (present) or 0 (absent). Results are expressed as the percentage occupied by fibrosis to the total area examined.

Reagents

Primary antibodies used were: anti-caspase-3, anti-SERCA-2a, anti-Kv1.5, anti-KCNE2, anti GAPDH and anti-vinculin. Secondary antibodies used were goat anti-rabbit IgG-AlexaFluor-488 (1:1000) and goat anti-mouse IgG-AlexaFluor-568 (1:1000) for immunofluorescence, goat anti-rabbit IgG-AlexaFluor-680 (1: 100,000, all from Invitrogen) and goat anti-mouse IgG-IR Dye 800 CW (1: 100,000, Odyssey, LI-COR) for Western Immunoblotting.

Statistical Analysis

Means were compared between the control and PH groups using t tests as all outcomes were well modelled by Gaussian (normal) distribution. A two-sided $P$ value less than 0.05 was considered statistically significant. Means and standard errors of the mean (SEMs) are reported.


Legends for Supplemental Figures

Figure 1. Selective downregulation of SERCA-2a protein in the RV of PH rats. Representative immunoblots of RV (A) and LV (B) from CTRL and PH-groups labelled with anti-SERCA2a and anti-GAPDH antibodies. Mean levels of SERCA2a protein normalized to GAPDH in CTRL (black bar) and PH (red bar) are shown at the right (*p<0.05 vs. CTRL, n=3-4 rats/group).

Figure 2. Snap shots during VF initiated in a heart isolated 29 days after MCT injection. Panel A shows snap shots of activation during 212 ms of VF during a period shown by the double-headed arrow in panel C, with arbitrarily chosen 0 ms in the first snap shot shown in panel A. Asterisks indicate focal activation sites and arrows show the direction of the propagating wave with incomplete rotating patterns. Panel B is a schematic OAP with red denoting depolarization and blue repolarization. Panel C shows seven consecutive OAPs recorded from the RV epicardial surface at sites indicated 1 to 7 with white arrow in snap shot 0 ms in panel A. Notice the irregular OAP reflecting mixed focal along with incomplete reentrant wavefronts and irregularly propagating wavefronts.

Figure 3. Direct effects of MCT on RV APD90. Bar graph showing mean action potential duration at 90% repolarization (APD90) in RV cardiomyocytes from normal healthy rats without (black bar) or with MCT (60mg/L) in the bath solution (red bar).

Legend for the Supplemental Movie

Movie showing activation wavefront dynamics during VF that becomes maintained in the failed RV by mixed focal and incomplete reentrant mechanisms.
Supplementary Figure 1 Umar et al
Supplementary Figure 3 Umar et al