Sildenafil Prevents and Reverses Transverse-Tubule Remodeling and Ca\textsuperscript{2+} Handling Dysfunction in Right Ventricle Failure Induced by Pulmonary Artery Hypertension

Yu-Ping Xie, Biyi Chen, Philip Sanders, Ang Guo, Yue Li, Kathy Zimmerman, Lie-Cheng Wang, Robert M. Weiss, Isabella M. Grumbach, Mark E. Anderson, Long-Sheng Song

Abstract—Right ventricular (RV) failure (RVF) is the main cause of death in patients with pulmonary artery hypertension (PAH). Sildenafil, a phosphodiesterase type 5 inhibitor, was approved recently for treatment of PAH patients. However, the mechanisms underlying RV contractile malfunction and the benefits of sildenafil on RV function are not well understood. We aimed to investigate the following: (1) the ultrastructural and excitation-contraction coupling alterations underlying PAH-induced RVF; (2) whether the ultrastructural changes are reversible; and (3) the mechanisms underlying the therapeutic benefits of sildenafil in PAH-RVF. We used a single injection of monocrotaline in Wistar rats to induce pulmonary vascular proliferation, which led to PAH and RVF. RV myocytes displayed severe transverse (T)-tubule loss and disorganization, as well as blunted and dys-synchronous sarcoplasmic reticulum Ca\textsuperscript{2+} release. Sildenafil prevented and reversed the monocrotaline-induced PAH and LV filling impairment. Early intervention with sildenafil prevented RV hypertrophy and the development of RVF, T-tubule remodeling, and Ca\textsuperscript{2+} handling dysfunction. Although late treatment with sildenafil did not reverse RV hypertrophy in animals with established RVF, RV systolic function was improved. Furthermore, late intervention partially reversed both the impairment of myocyte T-tubule integrity and Ca\textsuperscript{2+} handling protein and sarcoplasmic reticulum Ca\textsuperscript{2+} release function in monocrotaline-treated rats. In conclusion, PAH-induced increase in RV afterload causes severe T-tubule remodeling and Ca\textsuperscript{2+} handling dysfunction in RV myocytes, leading to RV contractile failure. Sildenafil prevents and partially reverses ultrastructural, molecular, and functional remodeling of failing RV myocytes. Reversal of pathological T-tubule remodeling, although incomplete, is achievable without the regression of RV hypertrophy. (Hypertension. 2012;59:355-362.) ● Online Data Supplement

Key Words: right ventricle failure □ pulmonary artery hypertension □ PDE5 inhibitor □ calcium □ T-tubule □ excitation-contraction coupling

Numerous previous studies on heart failure have focused on the left ventricle (LV). Our recent work and the work of others have demonstrated characteristic alterations in the myocyte transverse (T)-tubule membrane system and excitation-contraction (EC) coupling function in animal models of LV heart failure. Failing LV myocardium is characterized by T-tubule loss and disorganization; reduction in amplitude and synchrony of Ca\textsuperscript{2+} release; reduction in coupling gain function; reduction in sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} content; and alterations in Ca\textsuperscript{2+} handling proteins, including downregulation of SR Ca-ATPase and upregulation of Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger.\textsuperscript{1-6}

In contrast to LV failure, there is a paucity of research in right ventricular (RV) failure (RVF). Little is known about the mechanisms underlying RVF, although more attention has been paid to it recently.\textsuperscript{7-9} The RV is thought to be different from the LV in embryological origin.\textsuperscript{10} The crescent-shaped RV is much thinner than the LV in free wall and has greater compliance. Because of these differences, the RV is more sensitive than the LV to stresses, including afterload increase. For example, patients with systemic hypertension can compensate for increased afterload for many years, whereas adult patients with pulmonary arterial hypertension (PAH) often rapidly progress to RVF, and patients with untreated, severe PAH have a high rate of morbidity and a life expectancy of only 2 to 3 years.\textsuperscript{7}

PAH is the most severe form of pulmonary hypertension. RVF is the main cause of death in patients with PAH.\textsuperscript{11}
However, the cellular and molecular mechanisms underlying RV contractile dysfunction in PAH are not well understood. In the present study, we used a well-established monocrotaline (MCT)–induced PAH-RVF model in rats	extsuperscript{12} to investigate the structure-function relationship of RV myocytes in PAH-induced RVF. Sildenafil, a phosphodiesterase type 5 (PDE5) inhibitor, recently approved for the treatment of PAH, is associated with reduced pulmonary vascular resistance, improved exercise capacity, and increased survival in patients.\textsuperscript{13} Accordingly, the most important determinant of survival in PAH is RV function.\textsuperscript{14}

In the present study, we investigated the effects of sildenafil on PAH-induced RV dysfunction, using an MCT-induced PAH-RVF rat model and laser scanning confocal microscopy. One of the key questions that we are asking is whether alterations in ultrastructure and EC coupling function are reversible in PAH-induced RVF on therapeutic intervention.

**Methods**

An expanded Materials and Methods section is available in the online Data Supplement (please see http://hyper.ahajournals.org). Animal experiments were performed according to the protocol approved by the University of Iowa Institutional Animal Care and Use Committee. Male Wistar rats (Charles River Laboratory, Inc) weighing 175–200 g were given a single SC injection of monocrotaline (MCT; 60 mg/kg, Sigma-Aldrich) or the same volume of 0.9% saline.\textsuperscript{12} Rats receiving MCT were subdivided into 3 groups: MCT alone, SilMCT/D1 (treated with sildenafil beginning 1 day after MCT injection, daily, SC, 100 mg/kg per day), or SilMCT/D23 (treated with sildenafil beginning day 23 after MCT injection, for 2 weeks, daily, SC, 100 mg/kg per day). The MCT alone and SilMCT/D1 groups were euthanized at day 24 to 25 for histology, confocal imaging (Ca\textsuperscript{2+}, T-tubules), and Western blotting. The SilMCT/D23 group was euthanized 2 weeks after sildenafil treatment for histology, confocal imaging (Ca\textsuperscript{2+}, T-tubules), and Western blotting. The SilMCT/D23 group was euthanized 2 weeks after sildenafil treatment for histology, confocal imaging (Ca\textsuperscript{2+}, T-tubules), and Western blotting. Echocardiography, lung histology, confocal Ca\textsuperscript{2+} imaging of field stimulated Ca\textsuperscript{2+} transients in single isolated myocytes, in situ confocal imaging of myocyte T-tubule structure on intact heart, Western blotting assay of EC coupling proteins, and statistical methods are described in the online Data Supplement.

**Results**

**MCT-Induced PAH-RVF and Effects of Sildenafil in Wistar Rats**

A single injection of MCT (60 mg/kg) in rats caused massive remodeling of the pulmonary arteries. The pulmonary artery wall thickness, as examined at day 24 to 25, was increased by >3-fold over saline-treated controls, leading to the obstruction of the pulmonary arteries (Figure 1A, 1B, and 1E). In control rats, there was no tricuspid regurgitation. Pulmonary artery pressure (PAP) could not be estimated with echocardiography, whereas at day 24 to 25, all of the MCT-treated rats had high systolic PAP (82±6 mm Hg; n=18). These results confirm development of PAH in MCT-treated rats.

We next examined the ability of sildenafil to either prevent or reverse PAH. Sildenafil, 100 mg/kg per day, when given 1 day after MCT injection (SilMCT/D1), prevented PAH (Figure 1C). Only 2 of 14 rats in the SilMCT/D1 group had detectable systolic PAP (37 and 40 mm Hg). Pulmonary artery wall thickness was significantly reduced with early intervention (Figure 1C and 1E), suggesting that sildenafil prevents PAH development in MCT rats. Next, we administered sildenafil to MCT rats beginning at day 23 (SilMCT/D23), at which point RV dysfunction was evident by echocardiography, and treatment continued for 2 weeks. This delayed sildenafil intervention reversed MCT-induced pulmonary artery remodeling, similar to that of the SilMCT/D1 group (Figure 1D and 1E), and echocardiography detected PAP in only 1 of 8 rats in this group (30 mm Hg).

Echocardiography and morphometric data demonstrated that MCT-treated rats developed RV hypertrophy (Figure 2A through 2C), marked RV dilation (Figure S1, available in the online Data Supplement at http://hyper.ahajournals.org, and Figure 2D and 2E), and a severe decline in RV fractional shortening (Figure 2F). SilMCT/D1 prevented both RV hypotrophy and dilation and maintained RV systolic function (Figure 2). RV weight (ie, dry RV weight, dry RV/LV+septum) and RV wall thickness were not significantly different between the control and SilMCT/D1 groups (Figure 2A through 2C). Although pulmonary artery remodeling in the SilMCT/D1 group was reversed to levels similar to that of SilMCT/D23 group (Figure 1), SilMCT/D23 failed to reverse RV hypertrophy (Figure 2A through 2C). Surprisingly, SilMCT/D23 rats showed significantly better RV contractile function compared with MCT-treated rats (Figure 2F). A total of 62.5% (5 of 8) of rats in the SilMCT/D23 group had improved RV systolic function after 2-week sildenafil treatment. Their averaged RV fractional shortening was significantly improved from 15.6±3.4% to 26.9±3.5% (P<0.05). However,
echocardiographic evidence showed that acute exposure of sildenafil (before and 1 hour after a single-dose injection, 100 mg/kg) did not affect either LV or RV function in MCT-induced PAH-RVF rats (Figure S2 and S3). These data suggest that the improvement in RV function was not attributed to acute effects of sildenafil.

Interestingly, LV filling was impaired in the MCT group, as evidenced by reduction in LV end diastolic volume (Figure 3A), stroke volume (Figure 3B), and cardiac output (Figure 3C). Sildenafil (both SilMCT/D1 and SilMCT/D23) restored LV filling and cardiac output to normal levels (Figure 3A through 3C), because of normalization of PAH and RV afterload (Figure 2). Nonetheless, the LV ejection fraction remained unaffected by MCT and sildenafil treatments (Figure 3D).

Figure 2. Effects of monocrotaline (MCT) and sildenafil treatments on right ventricle (RV) morphometric and RV systolic function. A and B, RV weight was measured after 24 hours of drying at 37°C (A) and normalized to left ventricle (LV)+septum weight (B). N=5, 6, 7, and 5 hearts per group, respectively. C, RV wall thickness during diastole; D and E, RV dimensions during diastole (D) and systole (E). F, RV fractional shortening (FS; %), calculated as (RV dimension during diastole – RV dimension during systole) divided by RV dimension during diastole. N=11, 10, 9, and 8 per group, respectively. MCT-treated rats developed RV failure (RVF) with significant reduction in RV FS. Sildenafil early treatment prevented MCT-induced RVF with maintained FS. Sildenafil late treatment improved RV systolic function vs those of MCT alone. **P<0.001 vs saline control; †P<0.05; ††P<0.001 vs MCT group; ‡P<0.05 vs sildenafil, 100 mg/kg per day, when given 1 day after MCT injection (SilMCT/D1) group.

Sildenafil Normalized Ca2+ Handling Defects in RV Myocytes From MCT-Treated Rats

To investigate the cellular mechanism underlying PAH-RVF, we next measured intracellular Ca2+ with a laser scanning confocal microscope in isolated RV myocytes. RV myocytes from MCT-treated rats had smaller and slower Ca2+ transients compared with control RV myocytes (Figure 4). The

Figure 3. Monocrotaline (MCT)-induced pulmonary artery hypertension (PAH) impairs left ventricle (LV) filling. A, End diastolic volume; B, LV stroke volume; C, cardiac output. MCT-induced PAH-right ventricle (RV) afterload increase caused impairment of LV filling. Both early and late treatments with sildenafil normalized LV filling and output. D, LV ejection fraction (EF) was normal among all groups. ***P<0.001 vs saline control; ††P<0.05; †††P<0.01 vs MCT group. n=11, 10, 9, and 8 per group, respectively.
A slow Ca²⁺ transients in RV myocytes from the MCT-treated group were reflected by prolonged Ca²⁺ activation (time to peak; Figure 4F) and decay phase (time to 50% decay from peak transient; Figure 4G). These data indicate that PAH-RVF is associated with compromised SR Ca²⁺ release function. In contrast, RV myocytes from the SilMCT/D1 group displayed normal Ca²⁺ transients, suggesting that early treatment prevents the development of RV hypertrophy/failure and preserves cellular Ca²⁺ handling. Furthermore, we found that sildenafil treatment at a late stage (SilMCT/D23) partially improved Ca²⁺ handling (Figure 4E through 4G). However, the improvement of myocyte Ca²⁺ handling function is not likely attributed to the acute effect of sildenafil. Ca²⁺ transients in RV myocytes from MCT-treated rats were not altered by local perfusion of sildenafil (10 μmol/L, for 10 minutes; Figure S4), supporting the echocardiographic finding from acute sildenafil-treated MCT rats (Figure S2 and S3).

Sildenafil Prevented and Partially Reversed T-Tubule Remodeling in RV Myocytes From MCT-Treated Rats

The myocyte T-tubule system is an important determinant of cardiac EC coupling function. We next examined whether T-tubule remodeling occurs in the MCT-induced PAH-RVF model and whether this ultrastructural remodeling is reversible on therapeutic treatment. In the MCT-treated group, we consistently observed drastic T-tubule remodeling in RV myocytes in contrast to the highly organized T-tubule network in RV myocytes from control rats (Figure 5A and 5B).

As shown in a representative T-tubule image in Figure 5B, RV myocytes from MCT-treated rats lost a majority of T-tubules, resulting in a severe decrease in T-tubule power (TTpower; Figure 5E), an index of T-tubule integrity. Surprisingly, RV myocytes from the SilMCT/D1 group displayed normal T-tubule organization with similar TTpower to that of the control group (Figure 5C and 5E). RV myocytes from the SilMCT/D23 group had improved T-tubule integrity and organization compared with the MCT alone group (Figure 5D and 5E), suggesting that this ultrastructure alteration is reversible even with delayed sildenafil therapy. Histogram analysis of TTpower distribution further confirmed our findings on MCT-induced RV myocyte T-tubule remodeling and beneficial effects of sildenafil in this model (Figure S5). Collectively, our data demonstrate that improvements in Ca²⁺ transients in sildenafil-treated RV myocytes are associated with the prevention and partial reversal of T-tubule ultrastructure remodeling.

In addition, analysis of cardiomyocyte surface area from T-tubule images revealed that early sildenafil treatment (SilMCT/D1) fully protected RV myocytes from developing cellular hypertrophy, whereas late treatment failed to suppress hypertrophy in myocytes from the SilMCT/D23 group (Figure S6). These cellular data are consistent with our observations at the organ and tissue levels (ie, echocardiography and morphometric measurements; Figure 2).

Effects of Sildenafil on Ca²⁺ Handling Proteins in RV Myocytes

Our recent data and that of the Wehrens group indicate that junctophilin 2 is involved in T-tubule remodeling during...
cardiomyopathy. In comparison with control RV myocytes, junctophilin 2 was reduced by 35% in MCT-treated RV myocytes, unchanged in the SilMCT/D1 group, and partially restored by SilMCT/D23 treatment (Figure 6A). The trend in junctophilin 2 protein expression levels correlates with the RV T-tubule changes described above.

Myocyte Ca$^{2+}$ handling function is also associated with several important ion channels and transporters. We identified a significant reduction in Cav1.2 Ca$^{2+}$ channels (Cav1.2; Figure 6B) and sodium-calcium exchanger (Figure 6C) expression in the MCT-treated group compared with control. The marked reduction in Cav1.2 is likely to contribute to decreasing the Ca$^{2+}$ transient amplitude and reducing synchrony of SR Ca$^{2+}$ release (ie, prolonged time to peak) in RV myocytes from MCT-induced PAH-RVF rats. Consistent with the prevention/recovery in T-tubule integrity, Cav1.2 levels were normal or partially recovered by sildenafil treatments in SilMCT/D1 and SilMCT/D23 groups, respectively. SR-associated proteins RyR2 and phospholamban remained largely unchanged, and SR Ca$^{2+}$-ATPase was slightly but significantly downregulated in the MCT-treated group (Figure 6D through 6F). These data suggest that the molecular mechanism by which sildenafil improves myocardial function is, at least partly, through normalization of Ca$^{2+}$ handling protein expression.

**Discussion**

The pulmonary circulation is intimately coupled with RV function in health and disease. RV function is the most important determinant of survival in patients with PAH. Despite the significant advances in our understanding and clinical practice in PAH over the past decades, RVF remains the common fatal pathway and consequence of PAH. However, our understanding of RVF is limited because of a paucity of research in this area.

In this study, we used an established, MCT-induced PAH model to study the structure-function relationship of T-tubules and intracellular Ca$^{2+}$ in RVF. The major findings are as follows: (1) MCT-induced RVF manifested with marked RV hypertrophy, RV dilation and systolic dysfunction, blunted and dyssynchronous Ca$^{2+}$ transients, severe T-tubule loss and disorganization, and alterations in levels of Ca$^{2+}$ handling proteins; (2) sildenafil administered at an early stage prevented the development of PAH and RV hypertrophy and failure and preserved normal T-tubule ultrastructure, normal Ca$^{2+}$ transients, and expression of key Ca$^{2+}$ handling proteins; (3) when given at a delayed stage (with established RV hypertrophy and failure), sildenafil reversed MCT-induced PAH but not RV hypertrophy and partially restored RV contractile function, RV myocyte Ca$^{2+}$ transients, T-tubule ultrastructure, and Ca$^{2+}$ handling proteins; (4) sildenafil treatment corrected MCT-induced impairment of LV filling; and (5) sildenafil did not acutely affect RV contractile function in live animals and intracellular Ca$^{2+}$ handling in isolated RV myocytes of MCT-induced PAH-RVF rats. Our data provide evidence that T-tubule ultrastructural remodeling is reversible on therapeutic treatment, independent of cardiac hypertrophy remodeling.

Our recent data suggested that (LV) T-tubule remodeling may represent a key mechanism underlying the transition from hypertrophy to (LV) heart failure. Severe T-tubule loss leads to a significant reduction or redistribution in Cav1.2 Ca$^{2+}$ channels on T-tubule membrane and RyR2s on SR membrane, and, therefore, reduction in Ca$^{2+}$ transient amplitude and synchrony. In this study, we proposed that RV myocyte T-tubule disruption during PAH-induced RV remodeling is an important factor in the development of RVF. On the other hand, improvement of the RV myocyte T-tubule ultrastructure was anticipated to increase RV contractile function. Consistently, our Ca$^{2+}$ handling and echocardiographic results did support this notion. Future studies to further establish the temporal relationship between T-tubule disruption and changes in Ca$^{2+}$ handling in MCT-injected rats, for example, in the early stage, may
provide a better mechanistic understanding of T-tubule remodeling and Ca\textsuperscript{2+}/H\textsuperscript{+} handling dysfunction in heart disease.

Sildenafil was approved recently for use in patients with PAH. The beneficial effects of sildenafil or other PDE5 inhibitors in PAH are thought to result from relatively selective vasodilatory and antiproliferative effects on the pulmonary vasculature.\textsuperscript{19} Our data reveal a potent effect of sildenafil on pulmonary vascular remodeling, consistent with the literature.\textsuperscript{20} A recent article from Michelakis group\textsuperscript{19} provided evidence that PDE5 is increased in hypertrophied human RV myocardium, and acute inhibition of PDE5 improves RV myocyte contractility, suggesting a direct, acute effect of PDE5 inhibition on RV myocyte function in disease. However, our data showed that acute exposure of sildenafil did not increase contractile function of both ventricles (Figure S2 and S3), nor did it enhance SR Ca\textsuperscript{2+} release function in RV myocytes (Figure S4) from MCT-treated rats. Moreover, we showed that early treatment with sildenafil maintained normal pulmonary artery resistance and prevented afterload increase-induced RV structural (hypertrophy, wall thickness, and chamber dimension) and ultrastructural (T-tubule) remodeling, as well as functional alterations. In addition, our data from the late treatment group (SilMCT/D23) revealed a potential benefit of a PDE5 inhibitor on RV function, improving or reversing RV myocyte T-tubule remodeling and, therefore, Ca\textsuperscript{2+} handling in “advanced” disease. This represents a heretofore-unappreciated mechanism of sildenafil as a therapeutic for PAH-RVF disease. Finally, we believe that benefit of PDE5 inhibitor therapy for PAH and RV dysfunction is mainly through its chronic effects on decreasing RV afterload and ensuing RV structural and functional improvement.

Figure 6. Alterations in expression of Ca\textsuperscript{2+}/H\textsuperscript{+} handling proteins in right ventricle (RV) myocytes in response to sildenafil treatment. RV tissue lysates were extracted for Western blotting assay. Representative blots and quantitation of junctophilin 2 (JP-2; A), Cav1.2 (B), Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger (NCX1; C), RyR2 (D), phospholamban (PLN; E), and sarcoplasmic reticulum Ca-ATPase (SERCA; F) levels. *P<0.05 vs control; †P<0.01 vs control; ††P<0.05 vs monocrotaline (MCT); ‡P<0.01 vs MCT; ††P<0.05 vs sildenafil, 100 mg/kg per day, when given 1 day after MCT injection (SilMCT/D1) group. n=4 to 5 hearts for each group.
Sildenafil is effective in improving PAH-related RV dysfunction; however, it is still debatable whether sildenafil exerts its therapeutic effects via its action on the pulmonary vasculature (eg, afterload unloading) or by a direct antihypertrophic effect on RV remodeling. Kass and colleagues have published a number of studies suggesting that sildenafil prevents, arrests, and even reverses LV hypertrophy, fibrosis, and dilatation in mice subjected to LV pressure overload induced by T-aortic constriction. Recently, two independent groups examined whether sildenafil provides similar, direct protection against RV remodeling, as shown in LV by Kass and colleagues. Surprisingly, they both provided evidence that sildenafil does not prevent but even exacerbates RV hypertrophy in a pre-established RV hypertrophy model induced by pulmonary trunk artery banding, indicating that sildenafil prevents myocardial remodeling in PAH mainly through an indirect action via RV unloading. The discrepant effects of sildenafil on pressure-overload-induced LV versus RV hypertrophy could possibly be due to different mechanisms involved in the development of hypertrophy between the RV and LV. It is being increasingly recognized that, in addition to important differences in gene expression, embryology, and physiology, the RV and LV may have divergent responses to stress, including activation of different signaling cascades.

The beneficial effects of sildenafil on T-tubule remodeling, particularly in the setting of unimproved RV hypertrophy, suggest that unloading of RV afterload (because of sildenafil-induced normalization of PAP) may play an important role. Increasing evidence demonstrates the occurrence of reversal remodeling in failing hearts after mechanical unloading (eg, in patients after LV-assisted device surgery or lung transplantation). LV-assisted device surgery has been associated with improved cardiac function and a broad spectrum of reverse remodeling events in LV myocytes (eg, changes in morphology and at the cellular, biochemical, molecular, and transcriptional levels). Our finding of reverse remodeling of the T-tubule ultrastructure reflects a new layer of cardiovascular benefits on mechanical unloading in sildenafil-treated PAH-RVF rats. However, mechanical unloading is not always beneficial. Unwanted chronic mechanical unloading may lead to damage of the T-tubule system and defects in Ca2+ handling.

**Perspectives**

In patients with idiopathic PAH, the clinical course is determined by progressive loss of cross-sectional area in the pulmonary arterial tree and by RVF resulting from increased afterload. Sildenafil was introduced into the treatment armamentarium for PAH by virtue of its known vasodilator properties. Here in this study, we elucidated the mechanisms of RVF in a PAH model and uncovered a new mechanism by which sildenafil provides therapeutic benefit in PAH. Specifically, RV myocytes rapidly develop maladaptive, ultrastructural remodeling and EC coupling dysfunction in response to an increase in pulmonary arterial resistance, leading to RV contractile failure during PAH. Early intervention with sildenafil prevents PAH and preserves RV structure and function at the (sub)cellular and whole-organ levels. Late intervention promotes partially reversed structural and functional remodeling in RV failing myocytes. The prevention and repair of maladaptive changes to RV T-tubules appears to be an important benefit of sildenafil in PAH-RVF. Although therapeutic effects were observed when sildenafil was introduced late in the disease course, it appears that early intervention is required to prevent pathological cellular and ultrastructural remodeling of the RV, a finding with important translational implications.

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**Disclosures**

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**References**


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Sildenafil Prevents and Reverses T-tubule Remodeling and Ca\textsuperscript{2+} Handling Dysfunction in Right Ventricle Failure Induced by Pulmonary Artery Hypertension

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Extended Methods and Materials

MCT-induced PAH-RVF and sildenafil treatment. Animal experiments were performed according to the protocol approved by the University of Iowa Institutional Animal Care and Use Committee. Male Wistar rats (Charles River Laboratory, Inc.) weighing 175 g – 200 g were given a single subcutaneous injection of monocrotaline (MCT, 60 mg/kg, Sigma-Aldrich) or the same volume of 0.9% saline.\textsuperscript{1-3} Rats receiving MCT were subdivided into 3 groups: MCT alone, Sil\textsubscript{MCT/D1} (treated with sildenafil beginning one day after MCT injection, daily, s.c, 100 mg/kg/day); Sil\textsubscript{MCT/D23} (treated with sildenafil beginning day 23 after MCT injection, for 2 weeks, daily, s.c., 100 mg/kg/day). MCT alone and Sil\textsubscript{MCT/D1} groups were sacrificed at day 24-25 for histology, confocal imaging (Ca\textsuperscript{2+} and T-tubules) and western blotting. Sil\textsubscript{MCT/D23} group was sacrificed two weeks after sildenafil treatment for histology, confocal imaging (Ca\textsuperscript{2+} and T-tubules) and western blotting. The animal sudden death rates are 0%, 40%, and 0% for control, MCT, and Sil\textsubscript{MCT/D1}, respectively. The death rate in Sil\textsubscript{MCT/D23} group was 28.5% (4 out of 14) and 20% (2 out of 10) before and after sildenafil treatment, respectively.

Echocardiography. Light general anesthesia was induced using ketamine, 25 mg/kg i.p. The chest was shaved and warmed acoustic coupling gel was applied. 2D images were acquired in LV short- and long-axis planes with an 8-MHz sector-array probe, yielding 100 frames per second. LV mass, volumes, and ejection fractions were calculated using the bi-plane area-length method. RV end-diastolic dimension and end-systolic dimension were determined in the short-axis at the level of the LV cordae tendinae. Systolic pulmonary artery pressure (sPAP) was estimated using color
Doppler-directed continuous-wave spectral Doppler recordings, acquired via the tricuspid inflow view, as: sPAP = 4V^2, where V = maximum tricuspid regurgitant velocity in m/sec, and sPAP is expressed in mmHg.

**Lung histology.** After euthanasia was confirmed, lungs were perfused with 0.9% saline. A tracheotomy was performed and the lungs were inflated under gravity with 4% paraformaldehyde. The left lung was isolated and fixed under vacuum for 24 hours, followed by further fixation for 24 hours at 4°C. The lungs were dehydrated and embedded in paraffin. Five-micron sections were prepared, and then stained with hematoxylin and eosin. Vascular wall thickness was determined in vessels measuring under 500 µm in length. Five random vessels were chosen per section and length in µm measured from the external edge of the vessel to the lumen, at 5 points on each vessel. All measurements were made at 200X.

**Confocal Ca²⁺ imaging of field stimulated Ca²⁺ transients in single isolated myocytes.** RV myocytes were isolated from the RV free wall using standard enzymatic methods. Myocytes were loaded with the Ca²⁺ indicator fluo-4 AM (Invitrogen, 10 µM for 20 min) in normal Tyrode’s solution (mM: NaCl 137, KCl 5.4, HEPES 10, Glucose 10, MgCl₂ 1, NaH₂PO₄ 0.33, pH adjusted to 7.4 with NaOH), and washed with fluo-4 free Tyrode solution for another 20 minutes. In a recording chamber, cells were field stimulated at 1 Hz until steady state. Confocal line-scan imaging was performed by a Zeiss LSM 510 confocal microscope (Carl Zeiss MicroImaging Inc., Germany) equipped with an argon laser (488 nm) and a x63, 1.3 NA oil immersion objective. The optical pinhole was set to 1 airy disc (with axial resolution <1 µm). Line-scan images were acquired at sampling rate of 1.925 ms per line, along the longitudinal axis of the cell. Digital image processing was performed by using custom devised routines with IDL program (ITT VIS Inc., Boulder, CO). All Ca²⁺ measurements were performed at 35-36°C. In some experiments, Ca²⁺ transients were examined in RV myocytes from MCT-injected rats, before and after 10-minute sildenafil (10 µM) perfusion.

**In situ confocal imaging of myocyte T-tubule structure on intact heart.** Intact rat hearts were Langendorff-perfused at room temperature with Ca²⁺-free Tyrode’s solution (oxygenated with 95% O₂ and 5% CO₂ during experiments), containing 2.5 µM MM 4-64, a lipophilic fluorescence indicator of membrane structure (AAT BioQuest, USA) for 20 min. The hearts were then placed in the perfusion chamber attached on the stage of a confocal microscope, and perfused with indicator free / Ca²⁺ free solution (with continuous oxygenation). The membrane structure of epicardial myocytes was analyzed in situ by confocal microscopy. The optical pinhole was set to 1
airy disc (axial resolution <1 µm). Each T-tubule image frame contains 202 x 202 µm² area of myocytes. Ten to fifteen images from different locations of RV free walls were acquired. T-tubule images were analyzed offline with custom routines composed with IDL program, as recently described.6

**Western blotting assay of EC coupling proteins.** The rat RVs were harvested, quickly rinsed in PBS solution, immediately frozen in liquid nitrogen and stored at -80°C. Frozen tissues were homogenized in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM NaF, 1 mM Na₅VO₄, 5 mM EGTA, 5 mM EDTA, 0.5% Triton X-100, 0.5% deoxycholate, 0.1% SDS), containing protease inhibitors (Sigma, P8340). Tissue lysates were then centrifuged at 12,000 X g for 3 min to remove insoluble debris. Protein concentrations were determined using the Pierce BCA assay (Pierce, Thermo Scientific). Samples (10 µg) were separated by SDS-PAGE (10% acrylamide) and transferred to PVDF membranes. Primary antibodies that recognize JP-2 (Santa Cruz, sc-51313), Cav1.2 (Alomone Labs, ACC-003), NCX1 (Thermo Scientific, MA3-926), RyR2 (Thermo Scientific, MA3-916), SERCA (Santz Cruz, sc-8094), PLN (Thermo Scientific, MA3-922) and GAPDH (Cell Signaling, #2118) were used. HRP linked anti-goat IgG (1:5000) and anti-rabbit IgG (1:10000) were used to visualize bound primary antibodies with the SuperSignal chemiluminescence substrate (Pierce, Thermo Scientific). The protein bands were quantified using Image J software (version 1.43d).

**Statistics.** Data were expressed as mean ± SE. Student’s t test was applied when appropriate. p<0.05 was considered statistically significant.

**References**


**Supplemental Figures**

*Figure S1.* Echocardiography of diastolic short-axis images. LV: left ventricular cavity; IVS: interventricular septum; RV: right ventricular cavity. Double arrow indicates RV end-diastolic dimension. White bar = 2 mm.
Figure S2. Acute exposure of sildenafil does not alter cardiac (LV) function. A-D, Echocardiographic results of LV function in MCT-induced PAH-RVF rats, before (MCT/Control) and 1 hour after Sildenafil administration (single dose, i.p., 100 mg/Kg). No statistical difference was detected for all parameters. N=5 MCT rats.
Figure S3. Acute exposure of sildenafil does not change RV function either. A-D, Echocardiographic results of pulmonary artery pressure and RV function in MCT-induced PAH-RVF rats, before (MCT/Control) and 1 hour after Sildenafil administration (single dose, i.p., 100 mg/kg). No statistical difference was detected for all parameters. N=6 MCT rats.
Figure S4. Acute exposure of sildenafil does not alter the amplitude (A) and kinetics (B & C) of SR Ca\(^{2+}\) release in RV myocytes from MCT-induced PAH-RVF rats. Ca\(^{2+}\) transients were measured at baseline (MCT/control) and 10 minutes after local perfusion of sildenafil (10 µM). No statistical difference was detected for all parameters between the two groups. n=28 cells from 2 MCT rats.
Figure S5. Histogram distribution of $TT_{power}$. Histogram analysis of $TT_{power}$ of all T-tubule images acquired from different regions of RV free wall (10-15 images from each RV). A-B, MCT treated rats displayed a leftward shift in $TT_{power}$ (mode shifted from 2.3 of control to 1.4). C-Sil$_{MCT/D1}$ group maintained a similar histogram with the same mode as control. D, The histogram of Sil$_{MCT/D23}$ group was shifted to the 'right', in comparison to that of the MCT-treated group (with a mode shift from 1.4 to 1.7), indicating improvement of T-tubule ultrastructure by Sil$_{MCT/D23}$ treatment.
**Figure S6.** Analysis of cardiomyocyte surface area from T-tubule images. Early sildenafil treatment (Sil_{MCT/1}) fully protected RV myocytes from developing cellular hypertrophy in MCT-injected rats, but late treatment failed to suppress RV myocyte hypertrophy in rats of Sil_{MCT/23} group. n=188-223 cells for each group. ***, p<0.001 vs control; †††, p<0.01 vs MCT group (Note: only cells with complete visibility in T-tubule imaging field were measured for myocyte area).