Evolving Technology

A dynamic and chamber-specific mitochondrial remodeling in right ventricular hypertrophy can be therapeutically targeted

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Supplemental material is available online.

Objectives: The right ventricle fails quickly after increases in its afterload (ie, pulmonary hypertension) compared with the left ventricle (ie, systemic hypertension), resulting in significant morbidity and mortality. We hypothesized that the poor performance of the hypertrophied right ventricle is caused, at least in part, by a suboptimal mitochondrial/metabolic remodeling.

Methods/Results: We studied mitochondrial membrane potential, a surrogate for mitochondrial function, in human (n = 11) and rat hearts with physiologic (neonatal) and pathologic (pulmonary hypertension) right ventricular hypertrophy in vivo and in vitro. Mitochondrial membrane potential is higher in the normal left ventricle compared with the right ventricle but is highest in the hypertrophied right ventricle, both in myocardium and in isolated cardiomyocytes ($P < .01$). Mitochondrial membrane potential correlated positively with the degree of right ventricular hypertrophy in vivo and was recapitulated in phenylephrine-treated neonatal cardiomyocytes, an in vitro model of hypertrophy. The phenylephrine-induced mitochondrial hyperpolarization was reversed by VIVIT, an inhibitor of the nuclear factor of activated T lymphocytes, a transcription factor regulating the expression of several mitochondrial enzymes during cardiac development and hypertrophy. The clinically used drug dichloroacetate, known to increase the mitochondria-based glucose oxidation, reversed both the phenylephrine-induced mitochondrial hyperpolarization and nuclear factor of activated T lymphocytes (NFAT) activation. In Langendorff perfusions, dichloroacetate increased rat right ventricular inotropy in hypertrophied right ventricles ($P < .01$) but not in normal right ventricles, suggesting that mitochondrial hyperpolarization in right ventricular hypertrophy might be associated with its suboptimal performance.

Conclusions: The dynamic changes in mitochondrial membrane potential during right ventricular hypertrophy are chamber-specific, associated with activation of NFAT, and can be pharmacologically reversed leading to improved contractility. This mitochondrial remodeling might provide a framework for development of novel right ventricle–specific therapies.

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Although mechanisms of left ventricular (LV) heart failure are widely documented, right ventricular (RV) failure remains understudied, despite its high clinical importance. RV dysfunction is a major cause of morbidity and mortality in many conditions, including pulmonary arterial hypertension (PAH), congenital heart disease, and lung transplant surgery.[1,2] In response to

Abbreviations and Acronyms

DAPI = 4,6-diamino-2-phenylindole
DCA = dichloroacetate
LV = left ventricle/ventricular
LVH = left ventricular hypertrophy
NFAT = nuclear factor of activated T lymphocytes
PAAT = pulmonary artery acceleration time
PAH = pulmonary arterial hypertension
RV = right ventricle/ventricular
RVH = right ventricular hypertrophy
SMC = smooth muscle cells
TMRM = tetramethyl–rhodamine methyl ester

increased afterload (as seen in PAH), the thin RV of the normal adult heart hypertrophies but eventually quickly dilates and fails. There are, however, situations in congenital heart disease wherein the RV remains hypertrophied and compensated for years despite the development of PAH. These cases are typically seen when there is no involution of the physiologic neonatal RV hypertrophy (RVH) and the fetal morphology persists through adulthood. In contrast, the normal adult LV can develop hypertrophy and remain in a compensated state in response to an increase in its afterload (systemic hypertension) for decades. The relatively early failure of the RV in pulmonary hypertension explains largely the much worse survival of patients with PAH compared with patients with systemic hypertension; at the same time, this raises the exciting possibility that something in the neonatal hypertrophied RV (normal remodeling) offers superior function and protection compared with the acquired RVH in adults with PAH (abnormal remodeling).[3] The cause of this early failure remains unknown and understudied and explains the lack of RV-specific therapies.[1,2]

We have recently described that phosphodiesterase 5 inhibitors, such as sildenafil, may be RV-specific inotropes.[4] This theory is based on the fact that phosphodiesterase type 5 is selectively expressed in the myocardium of the hypertrophied RV but not in the LV of the same animal.[4] In the search for better RV-specific therapies, as opposed to the LV, identification of differences between the two ventricles is critical. There are several studies examining the metabolism of the LV,[5,6] but there is an impressive lack of studies on the metabolism of the RV. There is some evidence for differences between the metabolism of the RV and LV, at least in hypoxic animals.[7] Potential differences in the metabolism or molecular biology between the two ventricles are not surprising given the recent discovery that the two ventricles have a different origin at early embryogenesis of the heart; whereas the RV develops from the anterior heart field, the LV develops from the early heart tube.[8] It is therefore not appropriate to extrapolate findings or conclusions from the LV to the RV. Also, the adaptation of the RV to increased afterload may be regulated by mechanisms different from those in the LV.[9] The need to specifically study RV function and failure was recently recognized by the National Institutes of Health as a priority.[1]

In the neonatal heart, the RV is physiologically hypertrophied, in response to the high pulmonary vascular resistance in utero. However, after birth, the thickness of the RV eventually becomes only a third of that of the LV, as the pulmonary vascular resistance gradually decreases.[10] The physiologic hypertrophy in the neonatal RV might be regulated by a "fetal gene program,"[11] which might be reactivated (at whole or in part) in adult disease states. Fetal and adult cardiac hypertrophy are also characterized by a predominantly glycolytic phenotype,[5,6,12] which in the LV,[13,14] vascular biology,[15] or cancer[16] is associated with a resistance to apoptosis. This has not been studied directly in the RV. The fact that metabolism and apoptosis are both directly regulated by mitochondria[17] suggests that a potential mitochondrial and metabolic remodeling might be central to the regulation of RVH.

We hypothesized that there is a chamber-specific and dynamic mitochondrial remodeling during RVH, which might be associated with its suboptimal performance; reversal of this mitochondrial remodeling might be beneficial, improving RV function. We studied mitochondrial membrane potential, a surrogate for overall mitochondrial function and metabolism,[15-19] in human and rat hearts. We used confocal microscopy and tetramethyl–rhodamine methyl ester (TMRM), a positively charged dye that localizes at the most negative organelles in the cell, the mitochondria.[17] Mitochondrial hyperpolarization or depolarization is detected and quantified by an increase or decrease in TMRM fluorescence, respectively. We show that human and rat RVH is characterized by a dynamic increase in mitochondrial membrane potential (more hyperpolarized than that observed in the normal RV and LV) and that inhibition of this by the clinically used metabolic modulator dichloroacetate (DCA, an inhibitor of the mitochondrial pyruvate dehydrogenase kinase[20]) increases inotropy in the hypertrophied RV, but not in the normal RV. Our work has significant translational potential as DCA is being used in humans with mitochondrial diseases[21] and has recently been shown to reverse mitochondrial hyperpolarization, increase glucose oxidation, and reverse disease phenotype in both cancer[16] and PAH.[15]

Methods

Complete details are available online in the E-Supplement Methods section.

Permission from the University of Alberta committees on human ethics and animal policy and welfare was attained for all experiments on human and rat tissues, respectively.

Human Heart Tissue Samples

Human samples were acquired from patients undergoing surgery for congenital heart disease or transplantation at the University of

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Alberta Hospital. Excised ventricular tissue samples (free wall) were immediately placed on ice and stained with TMRM and Hoechst (a nuclear stain) for 40 minutes and visualized under confocal microscopy.[15,16,19] The presence of hypertrophy was documented by the use of echocardiography for every patient (Table 1) and confirmed macroscopically by the surgeon.

Animal Model of RVH

We studied RVH using a model of experimental PAH by injecting monocrotaline, an alkaloid from *Crotalaria spectabilis,* a widely established rat PAH model, as previously described[15,19] (E-Supplement Methods).

Isolation of adult rat cardiomyocytes. Adult Sprague–Dawley rats (300–350 g) were used and cardiomyocytes were isolated from the ventricles as previously described[4] (E-Supplement Methods).

Isolation and culture of neonatal rat cardiomyocytes. Neonatal Sprague–Dawley rat pups, 2 days old, were used to isolate RV and LV cardiomyocytes, which were then separated from fibroblasts and placed in culture, as previously described[22] (E-Supplement Methods). Immunocytochemistry for myosin heavy chain confirmed that the studied cultured cells were cardiomyocytes (Supplement Figure E1).

Staining and confocal microscopy of cells and tissues. TMRM was made up to a concentration of 20 nmol/L in plating media along with 0.5 μmol/L of Hoechst nuclear stain. Each 35 mm $\times$ 10 mm plate of cells received 2 mL of the staining solution for a period of 30 minutes at 37°C. For ventricular rat tissue, the exposure was 40 minutes. The staining media was then removed, and each plate was rinsed and left at 37°C in another 2 mL of plating media. Staining of plates was staggered to give each plate from each ventricle the same amount of exposure to TMRM and the same amount of time before imaging.

Immunohistochemistry and confocal microscopy were performed on a Zeiss LSM 510 multiphoton confocal microscope (Carl Zeiss, Inc, Jena, Germany) using antigen retrieval and Image enhancer IT (Invitrogen Corporation, Carlsbad, Calif) for nuclear factor of activated T lymphocytes (NFATc3) and 4,6-diamino-2-phenylindole (DAPI, a nuclear stain) as previously described[4,15,16,19,23] (also see E-Supplement Methods).

Isolated rat RV Langendorff perfusion. Adult rats with normal and hypertrophied heart (owing to monocrotline-induced PAH) were used. The heart were removed and perfused in a modified Langendorff preparation designed to study RV contractility, as we recently described[4] (also see E-Supplement Methods).

Statistics

Comparison between LV and RV cardiomyocytes (from either neonatal or adult models) was done with a *t* test. Analysis of ventricular tissue (from either adult rat or human) and neonatal cultured neonatal cardiomyocytes was completed via 1-way analysis of variance with post hoc Bonferroni correction.

Results

Dynamic and Chamber-specific Increase in Mitochondrial Membrane Potential in Human and Rat RVH

We first examined fresh human myocardial samples that were excised during surgical procedures within 30 minutes of excision. We obtained tissues from 11 patients with normal or hypertrophied RVs based on preoperative echocardiography (RV free wall thickness, Table 1). We were able to secure tissues from only two normal RVs and LVs. All specimens were loaded with TMRM and handled and imaged under identical conditions. There was increased TMRM fluorescence in the normal LV samples compared with the RV from the same patient (Figure 1, *A*). There was also increased TMRM fluorescence in RVH samples compared with the normal RV (Figure 1, *B* and *C*). Because we were able to secure only two normal hearts, direct comparisons among groups cannot be performed. However, results observed in the rat ventricular tissues were similar to the human RV and LV samples, where the normal adult rat LV had a higher mitochondrial membrane potential than the RV (Figure 2, *A;* $P < .01$). To determine whether this was due to differences in cardiomyocytes, rather than other cells (ie, fibroblasts), we isolated rat adult RV and LV cardiomyocytes. Indeed, the

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TABLE 1. Tissues obtained From 11 patients with normal or hypertrophied RVs

Patient	Age, sex	Diagnosis	Tissue	RV thickness (cm)
1	19 y, F	Left atrial sarcoma; heart transplant	Normal LV, normal RV	0.3
2	46, y M	RV failure after heart transplant; repeat heart transplant	Normal LV	—
3	54, y M	Intractable angina, no PAH, moderate LVH; heart transplant	Normal RV	0.4
4	4 m, F	Tetralogy of Fallot; surgical specimen	RVH	1.0
5	3 y, M	RV outflow tract obstruction; surgical specimen	RVH	1.1
6	5 m, M	Tetralogy of Fallot; surgical specimen	RVH	0.9
7	58 y, F	Rheumatic valve disease; heart transplant	RVH	1.6
8	11 d, M	Hypoplastic left heart; surgical specimen	RVH	0.7
9	2 y, F	Coarctation of aorta and ventricular septal defect; surgical specimen	RVH	1.0
10	6 m, M	Pulmonary stenosis and ventricular septal defect; surgical specimen	RVH	0.8
11	4 m, M	Tetralogy of Fallot; surgical specimen	RVH	0.9

RV, right ventricle; *LV,* left ventricle/ventricular; *PAH,* pulmonary arterial hypertension; *LVH,* left ventricular hypertrophy; *RVH,* right ventricular hypertrophy.

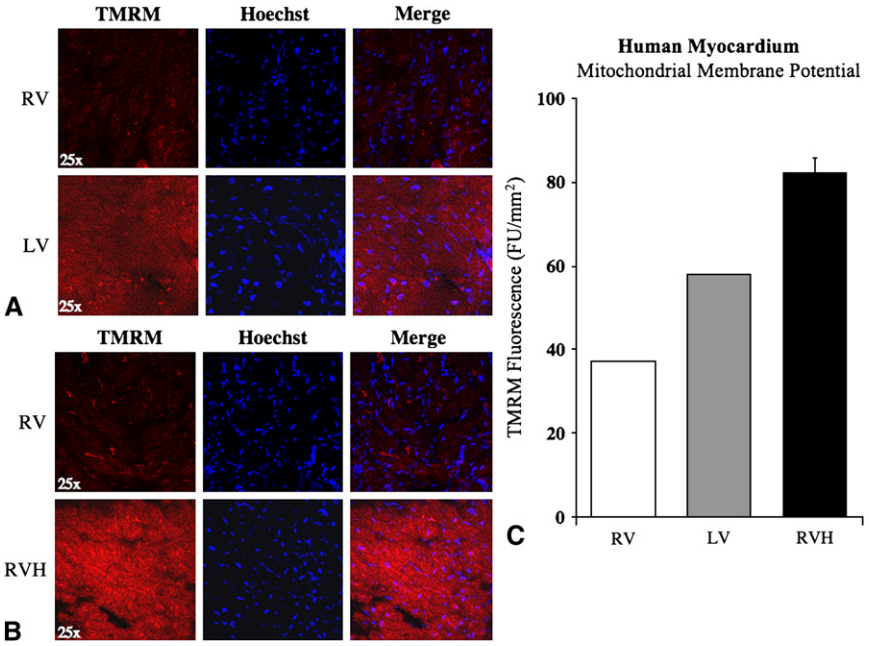

Figure 1. Differences in mitochondrial membrane potential between human RV and LV myocardium. A and B, Confocal microscopy images of acutely TMRM and Hoechst loaded normal RV and LV myocardium from patient 1 (A) and normal RV and RVH from Patients 3 and 6, respectively (B). C, Mean TMRM fluorescence from all patients expressed in fluorescence units per area (n = 2 RV, n = 2 LV, n = 8 RVH).

differences in mitochondrial membrane potential from whole myocardial tissue persisted at the level of individual cardiomyocytes (Figure 2, *B*). The mean TMRM fluorescence data showed a similar increase in myocardial and cardiomyocyte mitochondrial membrane potential in the LV versus the RV, respectively (Figure 2, *C*). Although these results are interesting descriptive findings between the two ventricles, the possibility to exploit these differences in targeted therapeutic strategies required further assessment of the changes that occur in disease states.

To examine the effects of hypertrophy on mitochondrial membrane potential, we used the model of monocrotaline-induced PAH and subsequent RVH in the rat. After injection with monocrotaline, the rat hearts were explanted on day 14 (moderate PAH and RVH) and day 28 (severe PAH and RVH). RV myocardium was loaded with TMRM and compared with vehicle-injected control animals (Figure 3). The worsening RVH was measured by echocardiography (free-wall thickness) and later confirmed macroscopically at autopsy (RV/LV + septum, data not shown). The increased

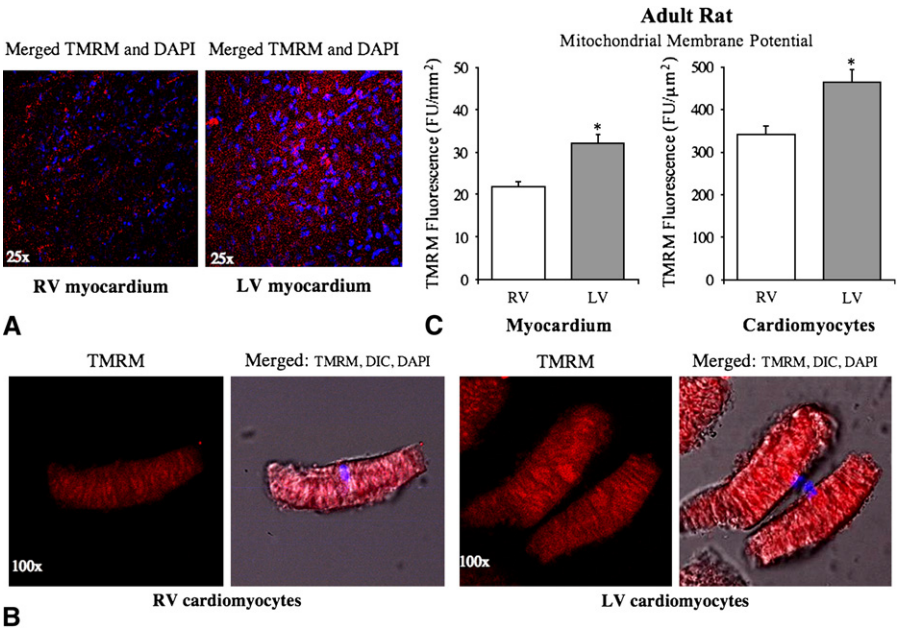

Figure 2. Similar mitochondrial membrane potential differences in adult rat RV and LV myocardium, also exist in isolated adult rat RV and LV cardiomyocytes. A, Confocal microscopy images of adult rat RV and LV myocardium loaded with TMRM and Hoechst. B, Isolated adult rat RV and LV cardiomyocytes loaded with TMRM and Hoechst. Merge panels also show differential interference contrast *(DIC)* channel for cell surface details. C, Mean TMRM fluorescence from all rat myocardial samples (n = 17 RV, n = 17 LV; *$P <$ .01 vs RV myocardium). Mean TMRM fluorescence for individual cardiomyocytes (n = 58 RV, n = 83 LV; *$P <$ .01 vs RV cardiomyocytes). *TMRM,* Tetramethyl–rhodamine methyl ester; *DAPI,* 4,6-diamino-2-phenylindole.

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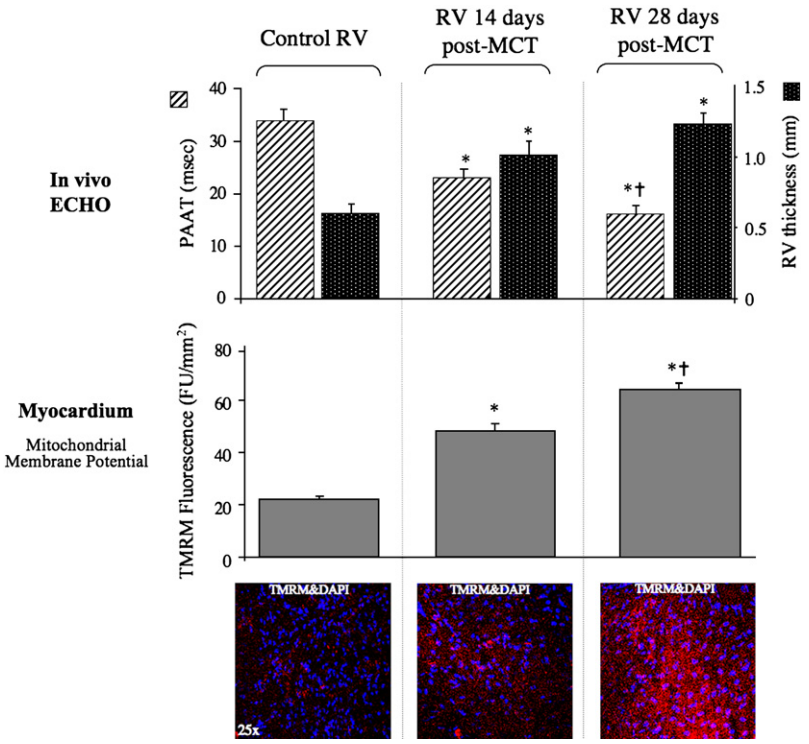

Figure 3. Mitochondrial membrane potential progressively increases with the development of pathologic RVH. *Upper third,* Pulmonary artery acceleration time *(PAAT; hatched bars)* progressively decreased from control to 14 days to 28 days after monocrotaline *(MCT)* injection, while this was mirrored by a subsequent increase in RV free wall thickness *(solid bars); *P < .01* versus control; †*P < .01* versus RV 14 days after MCT injection. *Middle third,* Mean TMRM fluorescence from all rat RV myocardial samples with progression of RVH after MCT injection (n = 17 control RV, n = 5 RV 14 days after MCT injection, n = 11 RV 28 days after MCT injection; *$P < .01$ vs control; †$P < .01$ vs RV 14 days after MCT injection). *Bottom third,* Representative confocal images of RV myocardium loaded with TMRM and Hoechst.

pulmonary artery pressure was shown by measurement of the pulmonary artery acceleration time (PAAT), which we[15,19] have previously shown to correlate negatively with mean pulmonary artery pressure in simultaneous right heart catheterization in rats. As hypertrophy progressed, there was a progressive increase in mitochondrial membrane potential in a "dose-dependent" manner (Figure 3).

Once again, to determine whether the cardiomyocytes develop mitochondrial hyperpolarization in hypertrophy and exclude possible effects of circulating factors in vivo, we used in vitro models. *Neonatal* cardiomyocytes (isolated from the neonatal RV, which is physiologically hypertrophied) can be cultured for 48 to 72 hours without significant change in phenotype, whereas isolated *adult* cardiomyocytes cannot be reliably sustained in culture.[22] We looked to mimic pressure overload hypertrophy by exposing cultured neonatal cardiomyocytes to phenylephrine. In this classic model of hypertrophy, in vitro cardiomyocytes have increased levels of intracellular calcium and develop hypertrophy within 48 hours.[22] Phenylephrine caused the predicted increase in cell size and a significant increase in mitochondrial membrane potential compared with vehicle-treated cardiomyocytes (Figure 4, *A*). The response to phenylephrine resulted in hyperpolarization of the mitochondria similar to that seen in physiologic RVH (723 ± 41 versus 587 ± 10 FU/mm^2, respectively; $P < .01$). Although it cannot definitely be excluded, it is unlikely that phenylephrine led to mitochondria biogenesis and an increased absolute number of mitochondria (which by itself would contribute to the increased TMRM signal), as the cells were only treated for 48 hours.

We then sought to determine a potential molecular mechanism for this mitochondrial remodeling and whether its normalization would lead to improved RV function.

Molecular and Metabolic Targeting of the Remodeled Mitochondria in Hypertrophy: The Role of NFAT

The increase in mitochondrial membrane potential in hypertrophy is likely multifactorial in etiology. First, there is an increase in intracellular and intramitochondrial calcium. This leads to activation of many mitochondrial enzymes that in turn cause an increase in Krebs cycle production of reducing equivalents, reactive oxygen species, and adenosine triphosphate, all of which alter mitochondrial function and mitochondrial membrane potential. Second, the increase in cytoplasmic calcium results in activation of the critical transcription factor NFAT,[24] which is activated and regulates anatomic and metabolic remodeling during heart development[25] and LV hypertrophy (LVH).[26] NFAT regulates the expression of many mitochondrial and metabolic genes (including adenylosuccinate synthetase 1,[27] pyruvate decarboxylase, heart–fatty acid binding protein, and the electron transport chain enzymes succinate dehydrogenase and cytochrome c oxidase[25]). NFAT is critical for heart development, as knockout of NFAT is fatal by embryonic day 10.5.[25] This transcription factor is highly conserved among species with the same isoforms found in humans and mice.[24] Thus, we

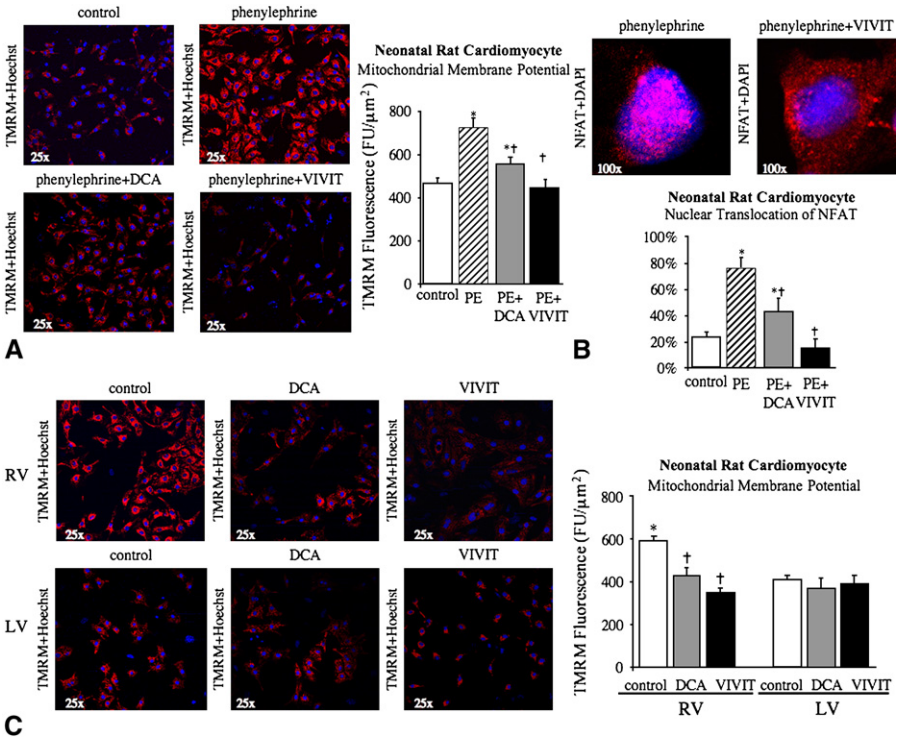

Figure 4. DCA and NFAT inhibition reverse the mitochondrial hyperpolarization seen in RV cardiomyocytes from physiologic (neonatal RV) and pathologic (phenylephrine-induced) hypertrophy in vitro. A, Representative confocal images of neonatal rat cardiomyocytes treated with vehicle, phenylephrine *(PE),* phenylephrine plus dichloroacetate *(PE+DCA),* or phenylephrine plus VIVIT *(PE+VIVIT)* and cultured for 48 hours before loading with TMRM and Hoechst. Mean TMRM fluorescence for individual cardiomyocytes (n > 140 cardiomyocytes per treatment; *$P < .01$ vs control; †$P < .01$ vs phenylephrine). B, Immunocytochemistry performed in fixed neonatal cardiomyocytes after phenylephrine or phenylephrine+VIVIT treatment show the prevention of nuclear factor of activated T lymphocytes *(NFAT, red)* translocation into the nucleus *(blue)* by VIVIT (n = 6 plates per group [>150 cells/plate]; *$P < .01$ vs control; †$P < .01$ vs phenylephrine). C, Isolated neonatal rat RV and LV cardiomyocytes treated with vehicle, DCA, or VIVIT and cultured for 48 hours before loading with TMRM and Hoechst. Mean TMRM fluorescence for individual cardiomyocytes (n = 473 LV, n = 449 RV; *$P < .01$ vs LV cardiomyocytes; †$P < .01$ vs control).

studied whether the increase in mitochondrial membrane potential was NFAT dependent by culturing neonatal cardiomyocytes with phenylephrine and VIVIT (a selective NFAT inhibitor[28]). VIVIT abolished the increase in mitochondrial membrane potential caused by phenylephrine to a level similar to that of untreated control neonatal cardiomyocytes (Figure 4, *A*). To confirm that VIVIT inhibited NFAT, we performed immunocytochemistry on fixed neonatal cardiomyocytes for NFATc3 (the isoform that has been studied the most in the heart) and DAPI (nuclear stain). NFAT activation is associated with a translocation of NFAT into the nucleus, whereas inhibition of NFAT with VIVIT restricts NFAT to the cytoplasm.[23,24] As expected, phenylephrine caused translocation of NFAT into the nucleus, whereas treatment with VIVIT inhibited this translocation and kept NFAT in the cytoplasm (Figure 4, *B*).

DCA inhibits the mitochondrial enzyme pyruvate dehydrogenase kinase, which in turn causes increased activity of pyruvate dehydrogenase, and thus DCA promotes the influx of pyruvate into the mitochondria, increasing glucose oxidation. Recently, DCA has been shown to reverse NFAT activation, mimicking the effects of VIVIT in cancer, increasing glucose oxidation, decreasing mitochondrial membrane potential in human cancer cell lines, and regressing tumor growth in vitro and in vivo.[16] DCA mimicked VIVIT and caused a decrease in mitochondrial membrane potential and inhibited the nuclear translocation of NFAT in the cultured neonatal cardiomyocytes, despite continued exposure to phenylephrine (Figure 4, *A* and *B*). Although we did not measure metabolism directly, DCA is known to increase the coupling of glycolysis to glucose oxidation in the postischemic heart, and although it does not have significant effects in the normal LV, it improves ischemia–reperfusion recovery in mild LVH.[29]

To determine whether DCA and VIVIT will depolarize mitochondria from physiologically hypertrophied hearts (as in the phenylephrine-induced hypertrophy) and whether this is ventricle-specific, we harvested neonatal rat hearts and isolated cardiomyocytes from separated RV (which is hypertrophied) versus LV (which is not hypertrophied) free walls. Similar to the adult RVH myocardium from monocrotaline-induced PAH, the isolated neonatal hypertrophied

cardiomyocytes from the RV free wall had more hyperpolarized mitochondria than those isolated from the LV free wall (Figure 4, *C*). Both DCA and VIVIT reversed this mitochondrial hyperpolarization and brought the mitochondrial membrane potential to the levels of the nonhypertrophied LV cardiomyocyte. Interestingly, DCA and VIVIT had no effect on the LV cardiomyocyte mitochondria (Figure 4, *C*).

These data show that in both physiologic and pathologic RVH, cardiomyocyte mitochondria are hyperpolarized, at least in part, due to NFAT activation, and that it can be reversed by DCA. Does this translate into improved RV function?

DCA Improves RV Inotropy in RVH

Since several patients with PAH or RVH owing to congenital heart disease only have affected RVs (their LVs are normal), the mitochondria-targeting DCA may selectively augment RV function in the setting of RVH. We used the ex vivo modified Langendorff perfused heart[4] to measure RV contractility (Figure 5, *A*). This modified model allows for real-time measurement of ex vivo developed pressures in the perfused RV, while its preload is constant (the balloon in the RV has a fixed volume) and is beating against no afterload (pulmonary artery transected). Interestingly, during perfusion of hearts 28 days after monocrotaline injection (severe RVH) compared with control animals, there was a significant and dose-dependent increase in developed pressure in the hypertrophied but not the normal RVs, whereas both had a similar increase in contractility by isoproterenol (Figure 5, *B* and *C*). Compatible with the expected DCA-induced increased glycolysis-to-glucose oxidation coupling, there was also an accompanied decrease in coronary effluent lactate only in the hearts with RVH (Figure 5, *D*).

Discussion

We describe dynamic and chamber-specific changes in mitochondrial function during the development of human and rat RVH, at the level of both the myocardium and the isolated cardiomyocyte (Figures 1 and 2). RVH is characterized in human and rat hearts by increased mitochondrial membrane potential compared with the normal RV, and this hyperpolarization appears to progress in parallel with the development of hypertrophy (Figure 3). We used monocrotaline-induced RV hypertrophy over pulmonary artery banding to better mimic acquired PAH in humans, where the RV is exposed to a gradual (not acute) rise in afterload. Even in the setting of physiologic RVH that occurs in the neonatal heart, RV cardiomyocytes have more hyperpolarized mitochondria than

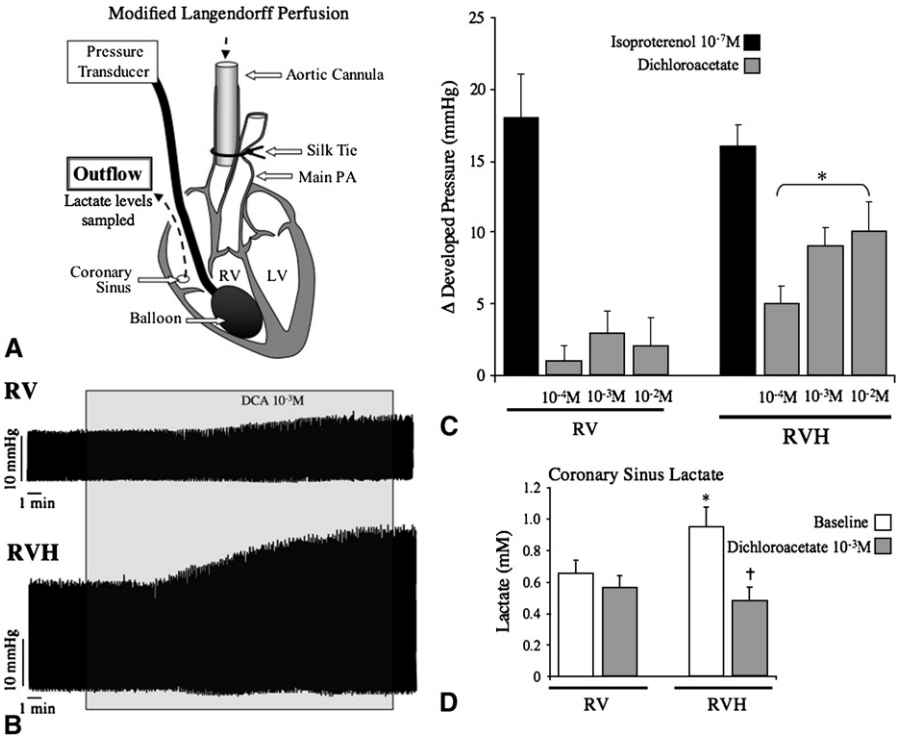

Figure 5. Targeted metabolic modulation by dichloroacetate *(DCA)* acutely improves RV inotropy in the ex vivo modified Langendorff perfused heart. A, Schematic of the isolated perfused RV (Langendorff preparation) to measure developed pressure. B, Representative real-time traces of the developed pressures from the normal RV *(upper)* and 28 days after monocrotaline injection RVH *(lower)* before and after the addition of DCA into the perfusate *(shaded area).* Note the significant increase in developed pressure only in the RVH hearts with acute DCA treatment. C, Mean data showing that DCA increased developed pressure (delta pressure from baseline developed pressure) in a dose-dependent manner in the RVH (n = 5) but not the normal RV (n = 6), whereas both normal and hypertrophied RV responded similarly to isoproterenol (*P < .01 vs normal RV). D, Coronary sinus effluent was collected on ice from the modified Langendorff perfusions 5 minutes after a steady baseline was established and 10 minutes after initiating perfusion with DCA and was analyzed for lactate concentration (n = 6 RV, n = 5 RVH; *P < .01 vs normal RV; †P < .01 vs baseline).

do LV myocytes (Figure 4, *C*). Cardiomyocyte hypertrophy can be induced in vitro by phenylephrine, which also leads to significant increases in mitochondrial membrane potential within 48 hours (Figure 4, *A*). Interestingly, this hyperpolarization is reversed by inhibition of a crucial transcription factor, NFAT, which has been shown to play a significant role in heart development[18] and LVH[26] but has not been previously studied in the RV hypertrophy (Figure 4, *A* and *B*). Furthermore, directly targeting mitochondria with DCA reversed the mitochondrial hyperpolarization in hypertrophy (Figure 4) and led to improved RV function (Figure 5). DCA did not affect the mitochondria and did not increase the contractile pressure of the normal RV. It is possible that the mitochondrial effects that we describe in RVH, both in vivo and in vitro, reflect a suboptimal mitochondrial remodeling, which may be responsible for suboptimal RV energy production and the relatively weak response of the RV to increased afterload. To the best of our knowledge, this is the first time in which the degree of hypertrophy is correlated to mitochondrial membrane potential and inotropic effects of a drug are related to the level of mitochondrial membrane potential, at least in the RV.

Although the RV and LV are currently approached and treated the same from a clinical perspective, the diversity of mitochondria between the RV and LV is not surprising given their different embryologic origins[8] and the increasing evidence for significant diversity of mitochondria among different organs, such as the liver and kidney.[30,31] The diversity of mitochondria extends beyond that of different organs to find diversity even within individual myocytes.[32,33] We[34] have previously shown that differences in mitochondrial function between the pulmonary and systemic arterial smooth muscle cells (SMC), where systemic SMC mitochondria are more hyperpolarized than pulmonary artery SMC mitochondria and account, at least in part, for the different response of the two vasculatures to hypoxia (the pulmonary arteries constrict whereas the renal arteries dilate). We subsequently demonstrated that, in PAH, the mitochondrial membrane potential in the pulmonary artery SMC is hyperpolarized compared with the SMC mitochondria from normal pulmonary artery SMC. We also demonstrated that treatment with DCA both normalized mitochondrial membrane potential and reversed PAH.[15,35] It is also intriguing that the proliferative pulmonary artery SMC from PAH patients also have activated (ie, nuclear) NFAT (like the RVH cardiomyocytes), whereas the normal pulmonary artery SMC smooth do not (like the normal RV cardiomyocytes).[23]

The molecular basis for mitochondrial remodeling in hypertrophy is unknown, although mitochondrial diversity among other organs is associated with varying degrees of electron transport chain complex expression, which needs to be explored between the RV and LV, as well as possible changes during hypertrophy from the normal ventricles. We provide preliminary evidence that NFAT might play an important role in this mitochondrial remodeling in RVH, similar to its recently described role in PAH[23] and cancer.[16] Our work cannot exclude the possibility that the increase in mitochondrial membrane potential is not due to an increase in mitochondrial number. However, the fact that a short-term exposure to DCA and VIVIT normalizes the mitochondrial membrane potential and the fact that DCA acutely improves RVH function suggests that the increased mitochondrial membrane potential has a functional basis.

The profile of DCA's effects in cancer and PAH (where it selectively increases apoptosis by depolarizing mitochondria) might raise concerns, inasmuch as it might increase RV apoptosis after long-term use. However, it is remarkable that long-term use of DCA has been shown to reverse PAH and RVH and to improve functional capacity and mortality in several animal PAH models.[15,16,36] It is possible that the DCA-induced mitochondrial depolarization in cardiomyocytes in the hypertrophied RV is enough to lead to improved contractile function but not enough to induce apoptosis by itself.

Furthermore, another medication that causes regression of PAH by inducing apoptosis in pulmonary artery SMC is sildenafil, which we[4] recently showed also increases contractility in RVH, much like DCA. However, long-term use of sildenafil (>2 years) in patients with PAH has not resulted in any cardiovascular related deaths and has led to improvement of RV function.[37] Nonetheless, this theoretical concern would need to be studied properly in the setting of a clinical trial.

The improved contractility of the hypertrophied RV with DCA is a novel finding. On the basis of the differences of the mitochondrial function between the two ventricles, we predicted that the effects of DCA would be restricted to the hypertrophied RV (and spare the normal RV and the LV). Indeed, DCA has failed to improve contractility from baseline in the LV, although there was a better recovery after ischemia.[29] The lack of improvement in LV contractility with DCA has also been shown in human studies where patients with coronary artery disease or congestive heart failure did not show improved LV contractility or cardiac output with short-term administration of intravenous DCA.[38,39]

Our findings that DCA improved RV contractility acutely might also be relevant to the many clinical conditions in which RV-specific inotropy is needed, including patients with postcardiotomy shock who have preoperative RV dysfunction, or in the surgery of pediatric patients with congenital heart disease and RVH.

Limitations

The study of mitochondrial membrane potential as a surrogate for mitochondrial metabolism is validated and accepted in the literature[35]; however, there are assumptions made using an in vitro model to represent in vivo findings. Most important, the in vitro environment of isolated cardiomyocytes does not mimic in vivo conditions, and although this is a confounding

factor, it is common to all isolated cells and we base our deductions from the delta membrane potential between the RV, LV, and treatment arms. Another limitation of the study is the small number of human samples obtained. However, we believe that these unselected human data are worthy of presentation because they are in agreement with our data from several in vivo and in vitro animal models, generally supporting the relevance of our hypothesis.

Conclusions

Selective agents targeting the diseased RV are highly desirable, given the poor performance of the RV in states of pressure overload (congenital heart disease, PAH, and post-transplant surgery). We provide evidence that therapeutically targeting mitochondria may allow for potential ventricle-specific treatments. This concept may help build the framework for a new class of drugs designed to affect only the diseased ventricle (ie, hyperpolarized mitochondria in RVH) while having little or no impact on the other more normal ventricle in the same patient. DCA is one such candidate therapy, which has minimal effects on normal cells throughout the body and does not significantly change the hemodynamics of normal animals[15]; this was also supported by our finding in the ex vivo perfused hearts, in which treatment with DCA did not have significant effects on the developed pressures generated by the normal RV (Figure 5). The documented effects of DCA in PAH, and now on RVH, raise the possibility of studying this drug in humans with PAH. The ability for a combined "double-hit" mechanism, wherein pulmonary vascular remodeling and PAH are reversed and at the same time RV function is directly enhanced, will be very desirable clinically. We recently described a similar mechanism in which sildenafil dilates the pulmonary circulation in PAH and at the same time directly increases RVH inotropy.[4] The fact that DCA has been used in humans with mitochondrial diseases for more than 30 years[21] supports the need for such phase II studies.

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Discussion

Dr Gordon Cohen *(Seattle, Wash).* Thank you for the opportunity to discuss this important presentation by Dr Nagendran and his colleagues at the University of Alberta. This paper was originally to be discussed by my department chief, Dr Edward Verrier. Unfortunately, Dr Verrier had to depart from the meeting early, so I will be discussing this paper in his place. Normally I would thank you for submitting the paper to me well in advance and allowing me plenty of opportunity to review it and think about it. Having just received the paper last night from Dr Verrier puts me in the unusual position of thanking you on his behalf for allowing him a lot of time to think about it, but my response to him is, "Thanks a lot, Ed, for the advance notice" [laughter].

This interesting set of experiments was designed to determine whether the mitochondrial metabolism of the RV, the LV, and the pathologically hypertrophied RV are distinct and unique. If so, the authors believed that they could represent specific targets for enhancing RV performance. This work is consistent with other investigations from this laboratory looking to identify mechanisms that could be pharmacologically exploited to improve performance in the struggling or failing RV. I have a few observations and question for Dr Nagendran.

First, why did you not study LVH as a more clinically relevant or prevalent problem? If nothing else, why did you not use LVH as a positive control? If your hypothesis is correct, then you might expect that an even greater change in mitochondrial membrane potential would be seen for LVH versus RVH.

Dr Nagendran. Thank you for your comments. The reason for specifically studying the RV is that most of the therapeutics that are used on the RV were only established in the LV, and models of LVH and changes in metabolism that occur in LVH have been documented. The changes that we show that are unique to other studies of LVH are the dramatic change in mitochondrial membrane potential and also coupling it to the changes in glucose oxidation when treated with DCA, which has already been checked in an LVH model. What we hope to show by these experiments is that there are inherently different methods of remodeling in the RV that may be less adaptive than the LV. To use LVH as a positive control is a very valid statement. The models for LVH require either invasive surgery, which would be aortic banding, or use of a spontaneously hypertensive rat that developed LVH, both of which introduced significant other variables to our model. That is why we studied the RV specifically.

Dr Cohen. Thank you for that explanation. Second, it appears to me that you are studying three different groups of cells, and I am not sure they all represent the same thing. First, you are looking at human RV muscle in which hypertrophy was documented by echocardiography. Presumably, this was an increase in RV wall thickness. In this group of patients, the increase in wall thickness presumably is something that developed slowly over time. One of the patients was undergoing a transplant, so presumably that was a long-term process. In the second group you studied rats. You had PAH induced with monocrotaline and then apparently humanely killed the rats at 2 or 4 weeks after again confirming an increase in RV wall thickness. Finally, you studied isolated neonatal rat myocytes that were exposed to phenylephrine for 2 days. Have you done any additional studies to ensure that you are not studying, for example, cellular edema at 48 hours in neonatal myocytes, increase in cell size at 2 weeks, and cellular hyperplasia plus or minus increase in cell size in the chronic state? If not, are you not concerned that you may be studying three different cellular states under the heading of RVH?

Dr Nagendran. Your point is well taken. The individual models of RVH are distinct. That is why in the monocrotaline-induced model, which is a pathologic model, we wanted to show correlation to a physiologic model, which is the neonatal model. In regard to whether these cells are different, when you treat with phenylephrine and induce hypertrophy, that does not mimic an in vivo–type experiment where you would have true pressure overload though the metabolic profile of all three types of hypertrophy were similar. That is the commonality between them that allows us to umbrella them under the category of RVH.

Dr Cohen. You looked at NFAT, which increases interleukin 2 activity during organ rejection. It does not appear, though, that you looked at any other transcription factors to see whether this was an NFAT-specific response. Was the response specific to NFAT or was the NFAT response part of some other more complex process that you observed?

Dr Nagendran. The reason for targeting NFAT specifically is that NFAT is critically linked to pathologic hypertrophy and cardiac

development. The reason why we chose to study NFAT is that in our other studies in cancer and in PAH, we showed a direct link of NFAT being inhibited by DCA. Once we saw the ex vivo perfusions and the return of function with DCA treatment, we honed in on similar mechanisms that were shown true in other organ systems. That is why we used NFAT, and indeed phenylephrine's primary method of causing hypertrophy in vitro is by an increase in intracellular calcium, which is the activating step for NFAT by binding to calcineurin.

Dr Cohen. Finally, how does DCA work in this setting? Is the DCA causing the mitochondria and the hypertrophied RV to function better, or is it recruiting more mitochondria? Did you do any studies to quantify the number of mitochondria present in the hypertrophied RV versus the normal RV? Is the change that you see in mitochondrial membrane permeability just a reflection of increased number of mitochondria that are present? What is the actual potential clinical utility of DCA? Numerous reports have described the compound as being neurotoxic.

Dr Nagendran. With regard to the number of mitochondria, we are also doing a concurrent study in which we examine the individual complexes along the electron transport chain. We remove the mitochondria from these cells and perform in vitro studies. There we find that the protein ratio of mitochondrial proteins to cardiomyocytes is similar in both the control and hypertrophy hearts. DCA caused hyperpolarized mitochondria to revert to a more normal depolarized phenotype after 2 days of treatment. However, in these data alone, we also have evidence that this reversion is a functional status rather than a change in actual number of mitochondria. The biogenesis of mitochondria is even more complex based on the fact that mitochondria undergo fission when increased energy demands are sensed, as well as undergoing fusion during times of stress to form rigid networks. Thus the absolute number of mitochondria might not change, while the functional state does.

Dr Cohen. Congratulations on an interesting study and thanks for the opportunity to discuss the paper.

E-Supplement Methods

All experiments on human tissues and rats were obtained with permission from the University of Alberta committees on human ethics and animal policy and welfare, respectively.

Human Heart Tissue Samples

Human samples were acquired from patients undergoing surgery for congenital heart disease or transplantation at the University of Alberta Hospital. Excised ventricular tissue samples (free wall) were immediately placed on ice and stained with TMRM and Hoechst (a nuclear stain) for 40 minutes and visualized under confocal microscopy.[E1-E3] The presence of hypertrophy was documented by the use of echocardiography for every patient (Table 1) and confirmed macroscopically by the surgeon.

Animal Model of RVH

We studied RVH using a model of experimental PAH by injecting monocrotaline, an alkaloid from *Crotalaria spectabilis,* a well-established model of rat PAH.[E2,E3] Monocrotaline is selectively toxic to the pulmonary arterial endothelium and causes significant RVH in 3 to 4 weeks after intraperitoneal injection. We[E2,E3] have repeatedly confirmed this using both invasive (right heart catheterization) and noninvasive (echocardiographic) methods. Here, we also confirmed the presence of PAH and RVH by echocardiography using PAAT (a parameter that correlates with mean pulmonary artery pressure and, used clinically, the shorter the PAAT the higher the pulmonary artery pressure) and RV free wall thickness in a short-axis parasternal view.[E2,E3] We further quantified RVH macroscopically at autopsy using the dry weight ratio of the RV/LV + septum.[E2,E3] Monocrotaline is activated in the liver and then absorbed in the pulmonary vascular bed without significant effects on systemic vasculature or cardiomyocytes. This concern has been examined by others, showing that all alterations to cardiomyocytes were specific to the RV owing to the developed PAH and not found in the LV of the same animals.[E4] Also, as monocrotaline requires activation by the liver, it is not possible to study the effects of monocrotaline in vitro.

Isolation of Adult Rat Cardiomyocytes

Hearts were taken from male adult Sprague–Dawley rats (300–350 g) and the aortas were cannulated and hung onto a perfusion system. Krebs buffer (NaCl, 118 mmol/L; KCl, 4.7 mmol/L; KH_2PO_4, 1.2 mmol/L; $MgSO_47H_2O$, 1.2 mmol/L; $CaCl_22H_2O$, 2.5 mmol/L; $NaHCO_3$, 25 mmol/L; glucose, 5 mmol/L) at pH 7.4 (corrected to temperature = 37.5°C) was antegradely perfused through the coronary arteries for 5 minutes. Perfusate solution was then switched to a 2% w/v collagenase (Worthington Biochemical, Lakewood, NJ) supplemented Krebs buffer for 20 minutes. The hearts were then removed from the system, and the two ventricular free walls were surgically separated for myocyte isolation. After another 5-minute digestion with 2% w/v collagenase, the samples were tritrated with a glass pipette filtered through a nylon mesh and the filtrate was centrifuged to yield cardiomyocytes.

Isolation and Culture of Neonatal Rat Cardiomyocytes[E5]

Hearts from 2-day-old rats were excised and atria were removed. Free walls of LV and RV were isolated and kept separately in phosphate-buffered saline. Ventricular tissue was minced in ice cold phosphate-buffered saline solution and then digested in an enzymatic cocktail containing 2% w/v collagenase, 0.5% w/v deoxyribonuclease (Worthington Biochemical), and 2% w/v trypsin (Worthington Biochemical) for 20 minutes at 37°C. After digestion, administering Dulbecco modified Eagle medium (DMEM)/F12 media (Sigma, Oakville, Ontario, Canada) supplemented with 20% fetal bovine serum (Sigma) stopped enzymatic reaction. The mixture was then centrifuged at 800 rpm for 1 minute at 4°C to remove fibroblasts, red blood cells, and debris in the supernatant. The pellet was redigested 2 to 3 times for another 20 minutes and centrifuged at 800 rpm to separate cardiomyocytes into the supernatant. The collected supernatant was finally pooled and centrifuged twice for 7 minutes at 1700 rpm to yield a pellet of cardiomyocytes. Owing to the quick attachment of fibroblasts to the plates compared with the rate of attachment of cardiomyocytes, we separate any existing fibroblasts in the mixture by removing the cardiomyocyte-rich supernatant from the fibroblasts attached to the plates (differential plating) for 2 hours at 37°C. The efficacy and specificity of this procedure have been previously validated by our group.[E5] The isolated neonatal rat cardiomyocytes were plated and maintained for 2.5 days in DMEM/F12 media supplemented with 5% fetal bovine serum, 10% horse serum (Invitrogen Canada Inc, Burlington, Ontario, Canada), and 50 mg/L gentamicin (Invitrogen). Media also contained 10 nmol/L cytosine arabinoside (Sigma) to prevent fibroblast proliferation. Cultured cells were treated for 48 hours with 10 μmol/L phenylephrine to induce cardiomyocyte hypertrophy as previously described.[E5] Phenylephrine-treated cells were also treated with either 5 mmol/L DCA or 4 mmol/L 11Arg-VIVIT (a competing peptide that selectively inhibits NFAT by blocking its binding to calcineurin[E6]) (EMD Biosciences, Mississauga, Canada); the high arginine content of the peptide significantly enhances its permeability into the cell.

Staining of Cells and Tissue

TMRM was made up to a concentration of 20 nmol/L in plating media along with 0.5 μmol/L of Hoechst nuclear stain. Each 35 mm × 10 mm plate of cells received 2 mL of the staining solution for a period of 30 minutes at 37°C. For ventricular rat tissue, the exposure was 40 minutes with tissues sliced between 140 and 160 μm in thickness. The staining

media was then removed, and each plate was rinsed once in media and then left at 37°C in another 2 mL of plating media. Staining of plates was staggered so as to give each plate from each ventricle the same amount of exposure to TMRM and the same amount of time before imaging. Imaging on confocal microscopy was performed at excitation of 543 nm, emission of 565 to 615 nm, and pixel scale of 0.9 mm × 0.9 mm. Background fluorescence was accounted for by usual techniques of setting the background to levels of control tissue that were not stained with TMRM. Photobleaching was not a significant factor inasmuch as regions where a short time course was obtained for quantifying TMRM fluorescence were only imaged once, avoiding the repeated imaging that causes photobleaching. Also, areas used for quantification of fluorescence were not imaged in high magnification, which also predisposes to photobleaching.

Immunohistochemistry and confocal microscopy were performed using antigen retrieval and Image enhancer IT (Invitrogen) for NFATc3 and DAPI (a nuclear stain), as previously described.[E1-E3,E7,E8]

Isolated Rat RV Langendorff Perfusion[E8]

Adult Sprague–Dawley rats (300–350 g) were anesthetized with intraperitoneal injection of 60 mg/kg pentobarbital. A midline sternotomy was performed, and within less than 30 seconds the heart was isolated and the aorta was cannulated and perfused with Krebs buffer at 12 to 13 mL/min. The hearts had a mean intrinsic rate of approximately 180 to 190 beats/min (hearts with a native rate <160 beats/min were not used). A latex balloon (Harvard Apparatus, Saint-Laurent, Quebec, Canada) was filled with water by titration to a constant volume of 30 mL and was placed in the RV via the right atrium through the tricuspid valve. The latex balloon was attached to a pressure transducer (Cobe, Richmond Hill, Ontario, Canada), and pressure traces were sampled at a rate of 1000 Hz by PowerLab data acquisition systems. Pressure readings were analyzed with Chart 5.4 software (ADInstruments Inc, Colorado Springs, Colo).

Imaging and Analysis of Data

All imaging was performed with a Zeiss LSM 510 confocal microscope. So that physiologic activity and viability of the tissue would be maintained, imaging was done on a heated platform at 37°C. Densitometry was analyzed with Zeiss Image Browser software. Fluorescence intensity of TMRM was assessed by measuring circular regions of interest (0.126 mm^2 in area). A region of interest was drawn into each field of view where the circle encompassed myocardial tissue only and not coronary vessels. For cardiomyocytes, the TMRM intensity was measured in each cell excluding the area containing the nucleus (as marked by Hoechst stain in blue) and divided by the cytoplasmic area. Phenylephrine-induced hypertrophy leads to increases in area cytoplasmic area by 35% to 50% after 48 hours of treatment in neonatal cardiomyocytes (n = 400–450), whereas in freshly isolated adult myocytes from animals with RVH, there was an increase of 12% (n = 56) in cell size compared with control adult RV cardiomyocytes.

Comparison between LV and RV cardiomyocytes (from either neonatal or adult model) was done using a *t* test. Statistical analysis of ventricular tissue (from either adult rat or human) and neonatal cultured neonatal cardiomyocytes was completed via 1-way analysis of variance with post hoc Bonferroni correction. All mean data are presented in bar graphs with standard errors representing variance.

E-References

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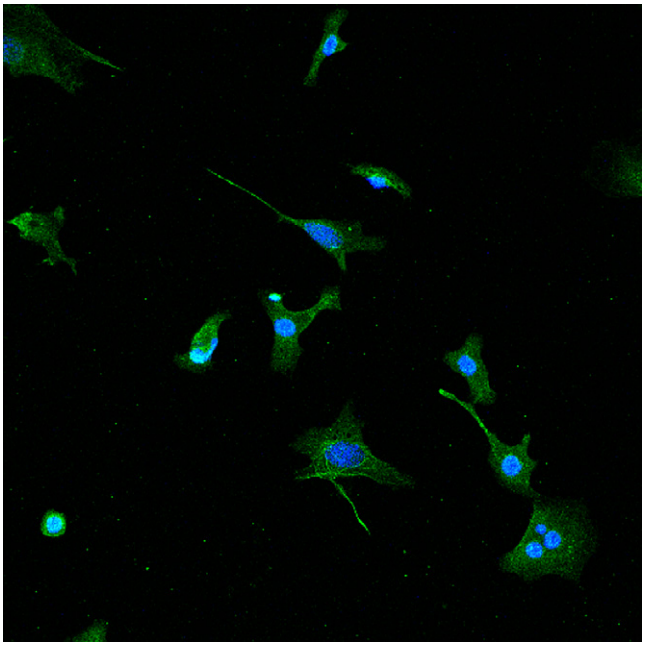

Figure E1. Verification of cardiomyocyte cell type. Representative confocal microscopy image of isolated neonatal rat cardiomyocytes stained for myosin heavy chain *(green)* and DAPI *(blue)* to confirm the cell isolation methods were specific for attaining cardiomyocytes over fibroblasts and/or vascular cells, with more than 97% of cells staining positive for myosin heavy chain.