Metabolic Gene Remodeling and Mitochondrial Dysfunction in Failing Right Ventricular Hypertrophy Secondary to Pulmonary Arterial Hypertension

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Background—Right ventricular (RV) dysfunction (RVD) is the most frequent cause of death in patients with pulmonary arterial hypertension. Although abnormal energy substrate use has been implicated in the development of chronic left heart failure, data describing such metabolic remodeling in RVD remain incomplete. Thus, we sought to characterize metabolic gene expression changes and mitochondrial dysfunction in functional and dysfunctional RV hypertrophy.

Methods and Results—Two different rat models of RV hypertrophy were studied. The model of RVD (SU5416/hypoxia) exhibited a significantly decreased gene expression of peroxisome proliferator-activated receptor-γ coactivator-1α, peroxisome proliferator-activated receptor-α and estrogen-related receptor-α. The expression of multiple peroxisome proliferator-activated receptor-γ coactivator-1α target genes required for fatty acid oxidation was similarly decreased. Decreased peroxisome proliferator-activated receptor-γ coactivator-1α expression was also associated with a net loss of mitochondrial protein and oxidative capacity. Reduced mitochondrial number was associated with a downregulation of transcription factor A, mitochondrial, and other genes required for mitochondrial biogenesis. Electron microscopy demonstrated that, in RVD tissue, mitochondria had abnormal shape and size. Lastly, respirometric analysis demonstrated that mitochondria isolated from RVD tissue had a significantly reduced ADP-stimulated (state 3) rate for complex I. Conversely, functional RV hypertrophy in the pulmonary artery banding model showed normal expression of peroxisome proliferator-activated receptor-γ coactivator-1α, whereas the expression of fatty acid oxidation genes was either preserved or unregulated. Moreover, pulmonary artery banding-RV tissue exhibited preserved transcription factor A mitochondrial expression and mitochondrial respiration despite elevated RV pressure-overload.

Conclusions—Right ventricular dysfunction, but not functional RV hypertrophy in rats, demonstrates a gene expression profile compatible with a multilevel impairment of fatty acid metabolism and significant mitochondrial dysfunction, partially independent of chronic pressure-overload. (Circ Heart Fail. 2013;6:136-144.)

Key Words: fatty acids ■ metabolism ■ mitochondria ■ pressure ■ pulmonary heart disease

Pulmonary arterial hypertension (PAH) is a severe and often rapidly progressive group of diseases that are characterized by a chronically and frequently progressive increase in the right ventricular (RV) afterload. Increased RV afterload is partially compensated by RV hypertrophy but eventually leads to RV dysfunction (RVD), RV failure, and untimely death, regardless of medical treatment. The prognostic importance of RVD in PAH, the cellular and molecular mechanisms underlying RVD need to be investigated, because they can be potentially reversible. Human and experimental chronic left heart dysfunction is characterized by decreased oxidative metabolism, abnormal mitochondrial respiration, and impaired mitochondrial biogenesis. These changes have in part been explained by a deregulated expression of critical transcription factors, such as the peroxisome proliferator-activated receptor (PPAR)-α, the estrogen-related receptor (ERR)-α, and the master regulator of oxidative metabolism, the PPAR-γ coactivator-1α (PGC-1α).

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Akin to left heart failure, it has been postulated that RVD is characterized by abnormal energy metabolism. Studies in animal models have demonstrated that RVD exhibits an increased expression of glycolysis-related genes and increased enzymatic glycolysis rate. However, it is largely unknown to what extent this switch in cardiac bioenergetics (also known as metabolic remodeling) involves changes in fatty acid oxidation (FAO), whether metabolic remodeling is an adaptive response to chronic pressure overload, or to what extent mitochondrial structure and function are also compromised in RVD. Thus, we sought to do the following: (1) characterize the metabolic gene expression profile associated with RVD; (2) determine whether pressure-overload is sufficient to explain metabolic gene remodeling; and (3) assess the structure and function of mitochondria in the dysfunctional right ventricle.

Here, we report that RVD, as assessed in rat and human RV tissue samples, is characterized by a downregulation of PGC-1α expression and decreased expression of several PGC-1α target genes encoding key enzymes that regulate FAO, as well as other genes involved in mitochondrial biogenesis. In addition, we present evidence for impaired mitochondrial structure and function (respiration). Some of these findings have been reported previously in abstract form.

Methods

Animal Models

**SU5416/Hypoxia Model (SuHx)**

An animal model of severe angioproliferative PAH and RVD was generated in male Sprague-Dawley rats (body weight, 200 g; age, 6 weeks) with a 20-mg/kg, 1-time subcutaneous injection of a vascular endothelial growth receptor blocker (SU5416), followed by 4 weeks of 10% hypoxia, as described previously and in the online-only Data Supplement. On return to normoxia, RV function was evaluated by transthoracic echocardiogram. The tricuspid annular plane systolic excursion was the reference parameter for RV function. For hemodynamic measurements, median sternotomy was performed, and RV systolic pressure was measured with a 4.5-mm Millar conductance catheter inserted at the RV outflow tract. As described previously, the RV in the SuHx rat model responds to pulmonary hypertension with a robust degree of hypertrophy, followed by dysfunction and failure. The RV in this rat model is characterized by fibrosis, capillary rarefaction, and cardiomyocyte apoptosis, which are associated with decreased cardiac output, markedly dilated RV, and decreased exercise capacity. As described previously, the SuHx RVD model reproduces some features of human RVD, such as paradoxical septalRV dilatation (online-only Data Supplement Movie I).

**Pulmonary Artery Banding**

Surgical ligation of the pulmonary artery was achieved through a left thoracotomy in male Sprague-Dawley rats weighing 180 to 200 g, with a silk suture tied around an 18-gauge needle alongside the pulmonary artery, as described previously (also in the online-only Data Supplement). The pulmonary artery banding (PAB) rats were killed to collect organs 6 weeks after the surgery to allow for significant hypertrophy, as reported by Bogaard et al. As a model of strictly mechanical RV pressure overload, the PAB rat model demonstrates preserved RV function despite generating significantly high RV afterload and hypertrophy (Figure S1F through S1H, available in the online-only Data Supplement). Thus, the PAB model was used as a model of nondysfunctional RV hypertrophy and was studied as above. The Table presents the echocardiographic and hemodynamic data illustrating the degree of pulmonary hypertension and RV function in all of the conditions studied.

Human samples were obtained from patients diagnosed with PAH who underwent cardiac transplantation. Oxygen consumption by intact mitochondria was measured with Clark-type oxygen electrodes. Immunohistochemistry and gene and protein expression studies were performed with standard procedures, as outlined in the online-only Data Supplement Methods section.

Statistical Analysis

Differences between groups were assessed with 1-way or 2-way ANOVA or Kruskal–Wallis tests. Bonferroni and Dunn post hoc tests were used to assess significant differences between groups. A $P$ value <0.05 was accepted as significant. Correlation analysis was done with a Spearman test. Results are reported as mean±SEM or fold-change mean±SEM unless specified otherwise. Four to 6 rats were used per group, unless otherwise specified. Statistical analysis was done with PASW V.18 (IBM, Armonk, NY) and GraphPad Prism (La Jolla, CA). For detailed information regarding echocardiography, hemodynamic measurements, gene expression, protein expression, human samples, mitochondrial isolation, measurement of mitochondrial respiration, chromatography electrospray ionization tandem mass spectrometry and immunohistochemistry, see the online-only Data Supplement.

Results

**RVD Is Characterized by a Load-Independent Downregulation of PGC-1α, PPAR-α, and ERR-α**

We have reported previously that dysfunctional RVs from SuHx-rats differentially express multiple gene signaling pathways when compared with nondysfunctional hypertrophied RVs. Importantly, we reported that RVD is associated with gene expression changes that suggest an abnormal metabolism, with a particularly strong signal relating to the PPAR signaling pathways. Therefore, as a first step, we measured the expression of PGC-1α, a direct coactivator and master regulator of the

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*Four to six rats were included per group: BW indicates body weight; HR, heart rate at the time of catheterization; LV, left ventricular weight; PAB, pulmonary artery banded; RV, right ventricular weight; RVSP, right ventricular systolic pressure; S, interventricular septum weight; SuHx, SU5416/hypoxia; and TAPSE, tricuspid annular planar systolic excursion measured by echocardiogram. Data are shown as mean±SEM.

†$P<0.05$ vs controls.
‡$P<0.0001$ vs controls.
§$P<0.05$ vs SuHx.
¶$P<0.05$ vs PAB.
PPAR family of transcription factors. PGC-1α regulates oxidative metabolism and many aspects of mitochondrial biology.21 Western blots of protein samples obtained from SuHx RV tissues showed a significantly decreased amount of PGC-1α protein (Figure 1A). Quantitative polymerase chain reaction analysis revealed a significant downregulation of PGC-1α mRNA levels (Figure 1B), indicating that the change in PGC-1α expression also occurred on the transcription level. Decreased PGC-1α gene expression was accompanied by a decreased expression of the ERR-α and PPAR-α genes (Figure 1B). To evaluate whether pure mechanical RV pressure-overload was sufficient to downregulate PGC-1α expression, we measured PGC-1α transcript levels in PAB-RV tissues and found that the expression of PGC-1α, ERR-α, and PPAR-α was not significantly decreased in the nonfailing hypertrophied RVs of PAB rats, despite the high RV pressure and RV hypertrophy (Figure 1B). Figures 1C and Figure S2A and S2B (available in the online-only Data Supplement) illustrate that PGC-1α (R²=0.72; P=0.001), PPAR-α (R²=0.72, P=0.001), and ERR-α (R²=0.76; P=0.002) transcript levels strongly correlated with the tricuspid annular plane systolic excursion, a heart rate–independent variable of RV function.

We have described previously that SU5416 treatment alone does not induce RVD and has a limited impact on gene expression.20 However, because decreased expression of PGC-1α could be a direct effect of the combination of SU5416 and hypoxia rather than a consequence of RVD, we measured the expression of PGC-1α 1 week after the SuHx protocol had been initiated. At 1 week no RVD is present and, indeed, as shown in Figure 1D, PGC-1α expression is not decreased in the right ventricle or in the left ventricle. In addition, we measured the expression of PGC-1α in rat lung tissues after 4 weeks of SuHx, a time point where plexiform-like lesions have already formed, and we found a significant increase in PGC-1α expression (Figure 1E). To further evaluate a potential toxic effect of SU5416 in the setting of RV pressure overload, we examined the RV gene expression of PAB animals exposed to SU5416 and found no change in the PGC-1α, ERR-α, or PPAR-α mRNA transcript levels (Figure S2C). Next, we measured the expression of PGC-1α in LV tissue.
obtained from animals killed 4 weeks after initiation of the SuHx protocol. Although somewhat decreased, the SuHx-LV tissue did not show a significant change in PGC-1α expression (Figure S2D). In the aggregate, the data indicate that the decreased expression of PGC-1α is unlikely attributed to a toxic effect of SU5416.

Because nuclear receptors other than PGC-1α have been implicated in phenotypic metabolic changes that occur during normal cardiac development, we decided to evaluate the expression of the ERR-γ and of the transcription factors COUP-TF1 and SP1. Quantitative polymerase chain reaction analysis showed that the expression of these transcription factors was not affected in dysfunctional RV tissues (Figure S2E). Lastly, to examine whether the changed expression of the metabolic gene pattern was also observed in human myocardium, we examined the gene expression of PGC-1α, PPAR-α, and ERR-α in RV tissue samples from patients with PAH. Indeed, we found a comparable expression pattern in the human RV tissue as we had observed in the SuHx RV rat tissue (Figure 1F).

**Dysfunctional RV Hypertrophy Is Characterized by Decreased Expression of Genes Involved in Fatty Acid and Glucose Oxidation**

Figure 2A illustrates that multiple PGC-1α/PPAR-α/ERR-α target genes encoding critically important proteins, required for fatty acid transport into the cardiomyocytes and into the mitochondria, were downregulated in the SuHx dysfunctional right ventricles. In addition, the expression of genes encoding proteins required for β-oxidation (acyl-coenzyme A [CoA] dehydrogenase, C-4 to C-12 straight chain [ACADM], acyl-CoA dehydrogenase, very long chain [ACADVL], and acyl-CoA dehydrogenase, C-4 to C-8(6) short chain [ACADS]) was decreased (Figure 2B). Western blot analysis confirmed the decreased expression of ACADM and ACADVL in RVD (Figure 2C). Supporting the observation of normal expression of PGC-1α/PPAR-α/ERR-α, nonfailing hypertrophied (PAB) right ventricles demonstrated a normal expression of ACADM and a significantly increased expression of ACADS and ACADVL. These acyl-CoA dehydrogenases are required to metabolize medium, short, and long fatty acids, respectively.

In accordance with our previous reports, dysfunctional RV hypertrophy in the SuHx rats was characterized by increased expression of GLUT1 and hexokinase 1; these 2 genes play an important role in glycolysis (Figure 2D). In contrast, we found a downregulation of genes that encode enzymes involved in aerobic glucose catabolism, such as the genes encoding the Krebs cycle enzymes citrate synthase and isocitrate dehydrogenase. Furthermore, the subunit of pyruvate dehydrogenase, an important link between glycolysis and glucose oxidation, showed a 50% decreased mRNA expression, whereas the gene expression of pyruvate dehydrogenase kinase, an enzyme controlling pyruvate dehydrogenase activity, was upregulated. Conversely, nonfailing hypertrophied PAB RVs exhibited a normal expression of glycolysis-related genes, a significantly lower expression of pyruvate dehydrogenase kinase, and normal expression of isocitrate dehydrogenase and citrate synthase (Figure 2D).

**RVD Is Characterized by Abnormal Mitochondrial Ultrastructure, Impaired Mitochondrial Respiration, and Abnormal Mitochondrial Biogenesis**

PGC-1α regulates mitochondrial biogenesis along with oxidative metabolism. Therefore, we sought to explore abnormalities in mitochondrial biology. PGC-1α exerts pleiotropic effects by direct coactivation of an array of nuclear and non-nuclear receptors used in the control of cellular metabolism. Among them, transcription factor A, mitochondrial (TFAM),
regulates mitochondrial DNA replication and maintenance and is required for cellular and mitochondrial viability.\textsuperscript{24,25} Associated with the decreased expression of PGC-1α, SuHx-RVD tissue had a decreased expression of TFAM (Figure 3A). Because reduced TFAM mRNA levels are associated with alterations in mitochondrial biogenesis, we measured the expression of Top1mt, POGL2, and POLRMT, a set of genes that encode enzymes required for the replication of mtDNA and mitochondrial biogenesis.\textsuperscript{4,25} All 3 of the genes were downregulated in dysfunctional (SuHx) RV hypertrophy but not in PAB-induced RV hypertrophy, as illustrated in Figure 3A. To test for decreased mtDNA transcription, we measured the expression of 2 mtDNA-encoded proteins, NADH-ubiquinone oxidoreductase subunit 4L and cytochrome B. These 2 proteins are subunits of the mitochondrial electron-transport chain complexes I and III, respectively. As illustrated in Figure 3B, the relative protein expression of NADH-ubiquinone oxidoreductase subunit 4L and cytochrome B was significantly decreased in SuHx-RVs.

High-power magnification electron microscopy demonstrated that the mitochondrial ultrastructure in RVD tissue was highly abnormal. In comparison to controls (Figure 3C), mitochondria in SuHx RVs were consistently abnormal in shape and size and clumped together in clusters (Figure 3D). Although clustering of mitochondria was also present in the PAB RVs (Figure 3E, arrow), the overall distribution of mitochondria was similar to that of control RVs. On isolation, RVD tissues exhibited a significantly decreased amount of mitochondria, as evidenced by mitochondrial yield and by citrate synthase activity (Figure 4A and 4B). Isolated mitochondria were studied by respirometry to evaluate the efficiency of oxidative phosphorylation. RVF mitochondria demonstrated a significantly decreased ADP-stimulated (state 3) respiration rate when using glutamate (Figure 4C) but not when using succinate as electron donors to complexes I and II, respectively (Table S2). The complete respirometry results are depicted in Table S2.

Because mitochondrial dysfunction could contribute to the generation of reactive oxygen species (ROS), we measured the levels of 8-isoprostan (8-isoProstaglandin F$_{2\alpha}$) in RVD tissues. 8-Isoprostan has been proposed as a marker of antioxidant deficiency and enhanced oxidative stress.\textsuperscript{26} Analysis from liquid chromatography-tandem mass spectrometry of SuHx RV tissue demonstrated no change in the amount of 8-isoprostan in comparison with controls (Figure S3A) but increased levels in the lungs (Figure S3B). However, although the amount of ROS generated might have not been sufficient to cause significant lipid peroxidation in whole RV tissues, ROS could still induce damage. mtDNA is particularly susceptible to ROS-induced damage,\textsuperscript{27} and a common marker of mtDNA damage is the formation of 7,8-dihydro-8-oxoguanine, a mutagenic base byproduct that regulates mitochondrial DNA replication and maintenance and is required for cellular and mitochondrial viability.\textsuperscript{24,25}
results from direct exposure of DNA to ROS. Figure 5 shows 8-oxo-G–positive staining in dysfunctional RV tissues, particularly in the endomyocardial area.

Discussion

RV failure is a common consequence of severe chronic pulmonary hypertension and the most frequent cause of death in patients with PAH. Although RVD plays an important prognostic role in patients with PAH, there are relatively few experimental data shedding light on the mechanisms of chronic RVD and failure. Although it has been proposed that RVD is associated with metabolic gene remodeling, a comprehensive metabolic gene profile of the failing right ventricle is still lacking.

Here we demonstrate that dysfunctional RV hypertrophy, in rats and patients with PAH, exhibits a significant reduction in the expression of PGC-1α and its corresponding nuclear receptors (PPAR-α and ERR-α). Interestingly, the change in PGC-1α expression seems to be largely independent of the RV pressure-overload and hypertrophy. Moreover, multiple PGC-1α target genes encoding proteins required for fatty acid metabolism were significantly decreased in expression in RVD tissues. Particularly, the expression of genes encoding the acyl-CoA dehydrogenases, which are specific for fatty acid β-oxidation, was significantly decreased in dysfunctional SuHx-RV hypertrophy but not in adaptive PAB-RV hypertrophy. Conversely, functional PAB-RV hypertrophy was associated with a high expression of ACADS and ACADVL, the latter being the most important heart acyl-CoA dehydrogenase for FAO. Altogether, the gene and protein expression data suggest that, in RVD, FAO is impaired on multiple levels. Along with the metabolic gene remodeling, we show evidence for an abnormal mitochondrial ultrastructure and decreased mitochondrial respiration at the level of complex I of the electron transport chain. Moreover, RVD is characterized by decreased expression of genes encoding proteins required for mitochondrial biogenesis, such as TFAM, Top1mt, POGL2, and POLRMT. RVD also demonstrated a significantly low mitochondrial yield in comparison with control RVs. Finally

Figure 4. A, Amount of mitochondrial protein per 100 mg of right ventricular (RV) tissue. SU5416/hypoxia (SuHx) and pulmonary artery banding (PAB) RV tissues demonstrate significantly decreased mitochondrial yield when compared with control RV tissue. B, Whole tissue citrate synthase activity assay demonstrates that SuHx has a reduced oxidative capacity when compared with control RV. C, State 3 respiration with complex I substrate (glutamate) is significantly decreased in SuHx RV dysfunction when compared with control or PAB RV tissues. The lines in the box-and-whiskers plots illustrate the median, whereas the + sign illustrates the mean.

Figure 5. Immunofluorescence shows increased 7,8-dihydro-8-oxoguanine (green) in right ventricular dysfunction (RVD) tissue vs SU5416/hypoxia (SuHx)-RVD.

Control RV

SuHx-RV
we demonstrate that RVD exhibits high levels of 7,8-dihydro-8-oxoguanine, consistent with ROS-induced DNA damage.

We decided to focus on central transcriptional regulators, such as PGC-1α and its corresponding nuclear receptors ERR-α and PPAR-α, because multiple gene knockout studies have illustrated that these proteins play an important role during the functional bioenergetic adaptation of the heart-to-pressure overload. PGC-1α is preferentially expressed in tissues with high oxidative capacity and coordinates several biological processes of mammalian energy metabolism by activating genes involved in cellular uptake and mitochondrial oxidation of fatty acids. Heart tissue obtained from PGC-1α knockout mice displays a reduced palmitoyl-l-carnitine state 3 respiration, suggesting reduced FAO, and a reduction in the amount of ATP generated per oxygen consumed. Of equal importance, in the absence of PGC-1α, the expression of mitochondrial genes in the heart is suppressed, the activities of mitochondrial enzymes are altered, and ATP production is reduced. As has been shown in models of left heart failure, the SuHx model of severe PAH and RVD is characterized by reduced PGC-1α expression (Figure 1A-B). We consider this reduced expression as a central component of RV metabolic remodeling. Although downregulation of PGC-1α is a feature of dysfunctional hypertrophy, it remains unclear what drives the downregulation of PGC-1α expression during RVD. Because the SuHx model is characterized by capillary rarefaction, ischemia and hypoxia could potentially drive the metabolic remodeling. However, PGC-1α expression is not decreased until RVD occurs. Because PGC-1α expression is a hypoxia-inducible factor–independent hypoxia-inducible gene, it is unlikely that the downregulated expression of PGC-1α and the associated metabolic remodeling profile would be entirely explained by hypoxia or by hypoxia-inducible factor activation. Although decreased PGC-1α mRNA expression has been reported in human left heart failure, recent studies using samples of left ventricles obtained from patients with heart failure have demonstrated a relatively normal expression of PGC-1α. Perhaps these discrepant results may be explained by different drug treatments of the patients with LV failure.

Although impaired glucose oxidation has been well characterized in the monocrotaline-injury model of RVD, changes in fatty acid metabolism are less clear. In our study, the downregulation of PGC-1α, ERR-α, and PPAR-α expression was coupled to a decreased expression of genes encoding fatty acid transport proteins and FAO, which suggests to us that fatty acid catabolism in the failing RVs is likely compromised on the levels of regulation, transport, and catabolism. Others have reported that changes in FAO occur in the monocrotaline-injury model of PH, mainly in carnitine palmitoyltransferase-1β expression, and few case reports have shown reduced uptake of radiolabeled fatty acid analogues in the RV of patients with PH. However, it remains unclear whether the changes in fatty acid metabolism are beneficial or detrimental in the overall function of the right ventricle. In the left ventricle, multiple studies have shown that the rate FAO is preserved or increased in physiological/adaptive hypertrophy and that FAO decreases during the progression of heart failure. Similarly, we demonstrate a normal/increased expression of ACADM, ACADS, and ACADVL in adaptive PAB-RV hypertrophy. These results are supported by the data of Fang et al, who demonstrated that rats with PAB-RV hypertrophy exhibit higher rates of FAO. We postulate that, along with capillary rarefaction, fibrosis, and ROS-induced damage, mitochondrial metabolic remodeling in RVD is pathological. It has been reported that FAO inhibition may have a therapeutic potential; however, it will remain to be tested whether further inhibition of FAO is beneficial in the model of SuHx RVD.

Although it has been reported that RVD is associated with mitochondrial hyperpolarization, a comprehensive analysis of mitochondrial respiration in RVD is still lacking. Here we demonstrate that RVD is associated with significant mitochondrial dysfunction, reduced mitochondrial yield, and reduced overall oxidative capacity. Surprisingly, although the expression of PGC-1α and TFAM was unchanged in PAB-RV hypertrophy, mitochondrial yield and citrate synthase activity were similarly decreased in both SuHx-RVD and PAB-RV hypertrophy. Because PGC-1α regulates mitochondrial biogenesis, our results would suggest an uncoupling between PGC-1α expression and mitochondrial biogenesis in adaptive PAB-RV hypertrophy. Yet, abnormal mitochondrial biogenesis can occur in the presence of normal PGC-1 levels. We speculate that preserved PGC-1α and TFAM expression could explain the better preserved mitochondrial respiration in PAB-RV hypertrophy when compared with SuHx-RVD, because both proteins play a critical role in mitochondrial function.

Study Limitations and Future Directions

We do not know whether a decrease of PGC-1α expression is a consequence or a cause of RVF. Nonetheless, our results show that decreased PGC-1α gene expression is not explained by a toxic effect of SU5416, hypoxia, or RV pressure overload. Several mechanisms may participate in the downregulation of PGC-1α expression in RVD and the associated gene metabolic remodeling. We did not explore whether the metabolic remodeling-dependent gene and protein expression profile affects enzymatic activity.

Conclusions

Our data illustrate that RVD is associated with a complex multilevel disturbance of FAO and mitochondrial respiration that is not entirely explained by pressure-overload or hypertrophy. We propose that RV metabolic remodeling is a consequence of decreased PGC-1α expression. To what extent metabolic remodeling or mitochondrial dysfunction is of functional importance for the development of RVD remains to be investigated.

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Disclosures

None.

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**CLINICAL PERSPECTIVE**

Our experiments demonstrate that in the setting of severe pulmonary arterial hypertension, load-independent alterations of cardiac energy metabolism and mitochondrial efficiency are associated with RVD. Because RV failure determines the outcome in patients with severe PAH, the cellular and molecular mechanisms that underlie RVF need to be understood, for they could be reversible. As we design RV-targeted therapies, the use of metabolic modulators should aim to restore not only substrate use but also mitochondrial function.
Metabolic Gene Remodeling and Mitochondrial Dysfunction in Failing Right Ventricular Hypertrophy Secondary to Pulmonary Arterial Hypertension


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SUPPLEMENTAL MATERIAL

Detailed Methods

Animal Models

SU5416/Hypoxia model
Male Sprague-Dawley rats (weight 200-250 g) received a single injection of SU5416 with a dose of 20 mg/kg subcutaneously and were exposed in a hypoxia (nitrogen dilution) chamber simulating an approximate altitude of 5000 m. for 4 weeks. The development of RV failure in this model is not attributed to a direct effect of SU5416 because: a) administration of SU5416 alone has no effect on either RV or LV; b) LV histology and function are normal in this model; c) the administration of SU5416 to rats with isolated mechanical pressure overload imposed by pulmonary artery banding is not associated with a decrease in RV function. (1)

Pulmonary artery banding
Male Sprague-Dawley rats (weight 180-200 g) were anesthetized with isoflurane inhalation (5% and 2%, respectively, in oxygen-enriched room air). After intubation, the animals are mechanically ventilated with the use of a volume-controlled respirator. Positive endexpiratory pressure was maintained at 4 cmH2O. A left thoracotomy as performed, where the pulmonary artery was carefully dissected free from the aorta. A silk thread was then positioned underneath the pulmonary artery, while we also placed an 8-gauge needle is placed alongside the pulmonary artery. A suture was tied tightly around the needle, and the needle rapidly removed to produce a fixed constricted opening in the lumen equal to the diameter of the needle. After the banding, the thorax was closed in layers, and postoperative pain relief was obtained by applying buprenorphine (15μg/kg s.c.). For more information about the procedure please refer to Bogaard et al (1).

Human samples
Human RV tissue samples were obtained from 4 patients diagnosed with PAH and RVF that underwent cardiac transplantation. All human samples were kindly provided by the Bosch Institute, University of Sydney, Sydney, Australia. Control RV samples were obtained from a
normal donor heart, carefully matching age and gender. Clinical information regarding the human samples can be found Supplemental Table 2.

**Echocardiography**

Doppler echocardiography was performed using the Vevo770 imaging system (VisualSonics, Toronto, Canada) directly after the hypoxic exposure (baseline). Superficial anesthesia with ketamine/xylazine was used to obtain two-dimensional, M-mode and Doppler imaging in both long axis and short-axis views, using a 30-MHz probe. The presence of pulmonary hypertension before treatment was evaluated by measuring the pulmonary artery acceleration time and mid-systolic notching with pulsed-wave Doppler recordings, as previously reported (2).

**Hemodynamic measurements**

Hemodynamic measurements were made using a 4.5 mm conductance catheter (Millar Instruments, Houston, TX) and the Powerlab data acquisition system (AD Instruments, Colorado Springs, CO). The rats were anesthetized with an intramuscular injection of ketamine at a dose of 100mg/kg and xylazine 15 mg/kg. Rats were then were intubated through tracheotomy and placed in a supine position. After a median sternotomy the RV outflow tract was punctured with a 23G needle and the catheter was introduced ante grade to measure pulmonary artery pressures.

**Gene expression studies**

Rat RV was carefully dissected from the LV and interventricular septum. Next, using the FastPrep®- 24 instrument (MP Biomedicals, Solon, OH), 25mg of tissue were homogenized with Triazol® (Qiagen, Valencia, CA) in Lysing Matrix D impact-resistant 2ml tubes (MP Biomedicals, Solon, OH). mRNA was carefully isolated using a RNeasy (Qiagen, Valencia, CA) isolation kit according to manufacturers protocol. Total RNA (1 μg) was reverse transcribed into complimentary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). First strand cDNA was diluted 10 times and RT-QPCR performed using Power SYBR® Green PCR Master Mix (Applied Biosystems) along with murine specific primers (Supplemental Table 3).
All PCR reactions were performed with a LightCycler480 PCR system (Roche Diagnostics, Meylan, France). The cycling parameters were the following: initial denaturation at 95°C for 15 min, followed by 50 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 15 s. The α-actinin 1 was used as the reference gene for mRNA relative quantification. All gene expression results were normalized to controls, and expressed as mean± SEM unless otherwise specified.

**Western Blotting**

Whole cell lysate from isolated right ventricle was prepared using RIPA (Radio-Immunoprecipitation Assay) buffer (Sigma, St. Louis, MO), and the protein concentration was determined using BioRad Protein DC Protein Assay (BioRad, Hercules, CA). Thirty micrograms of whole cellular protein per lane was separated by SDS-PAGE with a 4-12% Bis-Tris NUPAGE gel (MES SDS running buffer) and blotted onto a PVDF membrane. The membrane was incubated with blocking buffer (5% non-fat dry milk/PBS 0.1%/Tween 20) at room temperature for 1 hour. The membrane was then probed with the primary antibodies diluted in blocking buffer overnight at 4°C. Subsequently, membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody diluted 1:1000 in blocking buffer. Blots were developed with ECL (PerkinElmer, Waltham, MA) on GeneMate Blue Basic Autorad Films (BioExpress, Kaysville, UT). Blots were scanned and densitometry analysis was done with ImageJ (National Institutes of Health 1997-2011, Bethesda, MD; http://imagej.nih.gov/ij).

**Antibodies**

Rabbit anti-PGC1α antibody was acquired from Cell Signaling Technology, Inc., Beverly, MA. Rabbit anti-ACADM and ACADVL antibody were acquired from Abcam plc, Cambridge, MA. Rabbit anti-PPARα antibody, ND4L and CytB were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and rabbit anti-estrogen related receptor alpha (EPR46Y) was purchased from Millipore, Billerica, MA.

**Isolation of mitochondria** - Isolation of a single population of cardiac mitochondria was conducted as described previously (3), except that approximately 100 mg of right ventricle
tissue were used. Briefly, tissue was washed in a modified Chappell-Perry (CP) buffer (buffer CP1 at pH 7.4: 100 mM KCl, 50 mM MOPS, 1 mM EGTA, 5 mM MgSO₄·7H₂O, 1 mM ATP), dried with Whatman filter paper, weighed, minced and homogenized in CP1 buffer using a polytron tissue blender (Kinematica, Bohemia, NY). The homogenate was supplemented with 5 mg/g (wet weight) trypsin (#T0303, Sigma-Aldrich), incubated with stirring for 15 min at 4 °C followed by addition of 3 ml of CP2 buffer (CP1 buffer containing 0.2% BSA (#A7030, Sigma-Aldrich) to attenuate trypsin activity). Digested tissue was further homogenized by two strokes using a digital steady-stirring tight Teflon pestle/glass tube homogenizer set at 600 rpm (Fisher Scientific, Pittsburgh, PA). Undigested tissue and heavier cell fractions in the remaining volume were pelleted by centrifugation at speed 500g for 10 min at 4 °C. The mitochondria-containing supernatant was centrifuged at 3000g for 10 min at 4 °C. The mitochondrial pellet was washed with 1 ml of KME buffer, pH 7.4 (100 mM KCl, 50 mM MOPS, 0.5 mM EGTA). Mitochondria were re-suspended in KME and used within 4 h after isolation or frozen. The protein concentration was measured by Lowry (4) using BSA as a standard and sodium deoxycholate as a detergent.

**Mitochondrial oxidative phosphorylation** - Oxygen consumption by intact mitochondria was measured using a Clark-type oxygen electrode (Strathkelvin Instruments, North Lanarkshire, UK) at 30 °C in respiration buffer at pH 7.4 (80 mM KCl, 50 mM MOPS, 1 mM EGTA, 5 mM KH₂PO₄, 1 mg/ml defatted BSA) as previously described (5,6). Briefly, substrates for complex I (20 mM glutamate), complex II (20 mM succinate with 7.5 μM rotenone), and complex IV (1 mM N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD)/20 mM L- ascorbate with 7.5 μM rotenone) were used and state 3 (0.2 mM ADP-stimulated), state 4 (ADP-limited) respiration, respiratory control ratio (RCR), maximal rate of state 3 respiration (2 mM ADP), and ADP/O ratio were determined.

**Chromatography Electrospray Ionization Tandem Mass Spectrometry**

Eicosanoids were analyzed from frozen heart samples as follows. Briefly the frozen heart tissues were thawed on ice and homogenized using an Omni TH tissue homogenizer to obtain a 10% (w/v) solution in PBS. 200 μl of the solution thus obtained was diluted 1ml of LCMS
grade ethanol containing 10 ng of each internal standard and 0.05% BHT. The internal standards used were, (d4) 6k PGF1α, (d4) 8-iso PGF2α, (d4) PGF2α, (d4) PGE2, (d4) PGD2, (d4) LTB4, (d4) TXB2, (d4) LTC4, (d5) LTD4, (d5) LTE4, (d8) 5-hydroxyeicosatetraenoic acid (5HETE), (d8) 15-hydroxyeicosatetraenoic acid (15HETE), (d8) 14,15 epoxyeicosatrienoic acid, (d8) Arachidonic Acid, and (d5) Eicosapentaenoic acid. The mixture thus obtained was agitated to homogeneity using a bath sonicator and the resultant suspension was incubated in the dark at 4°C for 5 hours with periodic sonication. Following incubation, the insoluble fraction was precipitated by centrifuging at 6000g for 20 minutes and the supernatant was transferred into a new glass tube. This supernatant was dried under vacuum followed by reconstitution in 100 μl of 50:50 EtOH:dH2O for quantitation via LC/MS/MS. A 30 minute reversed-phase LC method utilizing a Kinetex C18 column (150 x 2.1mm, 1.7µm) was used to separate the eicosanoids at a flow rate of 200μl/min at 50°C. The column was equilibrated with 100% Solvent A [acetonitrile:water:formic acid (10:90:0.02, v/v/v)] for five minutes and then 10 µl of sample was injected. 100% Solvent A was used for the first minute of elution. Solvent B [acetonitrile:isopropanol (50:50, v/v)] was increased in a linear gradient to 25% Solvent B to 3 minutes, to 45% until 11 minutes, to 60% until 13 minutes, to 75% until 18 minutes, and to 100% until 20 minutes. 100% Solvent B was held until 25 minutes, then was decreased to 0% in a linear gradient until 26 minutes, and then held until 30 minutes. The eicosanoids were analyzed using a hybrid triple quadrupole linear ion trap mass spectrometer (QTRAP5500® ABSciex) via multiple-reaction monitoring in negative-ion mode. Eicosanoids were monitored using precursor → product MRM pairs. The mass spectrometer parameters used were: curtain gas: 30; CAD: High; ion spray voltage: -3500V; temperature: 500°C; Gas 1: 40; Gas 2: 60; declustering potential, collision energy, and collision cell exit potential vary per transition.
**Supplemental Table 1.** Demographic and clinical information of patients with right ventricular dysfunction and pulmonary hypertension. Heart samples from free RV wall were obtained after cardiac transplantation, immediately frozen and stored. NYHA = New York Heart Association Functional Classification at the moment of cardiac transplantation; EMS = Eisenmenger’s Syndrome; IPAH = Idiopathic Pulmonary Arterial Hypertension; VSD = Ventriculoseptal defect

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<th>Patient</th>
<th>Age</th>
<th>Gender</th>
<th>NYHA Class</th>
<th>Clinical Data</th>
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<td><strong>Control</strong></td>
<td>42</td>
<td>Female</td>
<td>I</td>
<td>Brain death due to subarachnoid hemorrhage. No previous cardiac or pulmonary disease.</td>
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<td><strong>EMS 1</strong></td>
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<td>Female</td>
<td>II</td>
<td>Large VSD and pulmonary hypertension.</td>
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<td><strong>EMS 2</strong></td>
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<td><strong>PAH 1</strong></td>
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<td>Familiar IPAH, mean pulmonary pressure 80 mmHg, wedge pressure 10, cardiac index 1.76. Negative vasoreactive test. No specific PAH treatment.</td>
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### Supplemental Table 2. Mitochondrial Respirometry Results

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<th>Group</th>
<th>Tissue input (mg)</th>
<th>Protein yield (mg/g of wet tissue)</th>
<th>State 3 (nA/min/mg)</th>
<th>State 4 (nA/min/mg)</th>
<th>RCR</th>
<th>ADP/O</th>
<th>2mM ADP (nA/min/mg)</th>
<th>DNP (nA/min/mg)</th>
<th>State 3 (nA/min/mg)</th>
<th>State 4 (nA/min/mg)</th>
<th>RCR</th>
<th>ADP/O</th>
<th>2mM ADP (nA/min/mg)</th>
<th>DNP (nA/min/mg)</th>
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### Supplemental Table 3. Primer sequences

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Supplemental Figure 1. Microphotographs showing the histological differences in the lung vasculature between controls (A), SuHx (B) and Pulmonary Artery Banding (PAB) (C). Figures F-H shows the differences in TAPSE, RVSP and hypertrophy (RV/LV+S) respectively. N= 4-10 per group. Data are shown in mean ± SEM. * p=<0.05, #p=<0.01
Supplemental Figure 2. Scatter-plot illustrating correlation between TAPSE and mRNA expression of ERR-α (A) and PPAR-α (B). RT-PCR analysis reveals no difference in the gene expression of PGC-1α, PPAR-α or ERR-α in the PAB model after exposure to SU5416 (C) or in SuHx left ventricle tissue (D). E) mRNA transcript levels of transcription factors other than PGC-1α that regulate cardiac energetics. N=4-8 per group. Data are shown in fold-change mean ± SEM. * p<=0.05, #p<=0.001
Supplemental Figure 3. LC-tandem mass spectrometric measurement of 8-iso-PGF-2α (8-isoprostane) in the RV (A) and lung (B) tissue of SuHx rats. N= 4 rats per group. There were no statistically significant differences between groups.

Legends for Supplemental Video

Supplemental Video 1. This video clip was obtained while performing an echocardiogram of a rat exposed to SU5416/hypoxia (RVD model). The clip illustrates the degree of RV dysfunction. Note the significant dilatation of the RV, the paradoxical septal movement and the important restriction of the left ventricle during systole and diastole.
Supplemental References


