Increased Reactive Oxygen Species, Metabolic Maladaptation, and Autophagy Contribute to Pulmonary Arterial Hypertension–Induced Ventricular Hypertrophy and Diastolic Heart Failure

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Abstract—Pulmonary arterial hypertension (PAH) is a debilitating and deadly disease with no known cure. Heart failure is a major comorbidity and a common cause of the premature death of patients with PAH. Increased asymmetrical right ventricular hypertrophy and septal wall thickening compress the left ventricular cavity and elicit diastolic heart failure. In this study, we used the Sugen5416/hypoxia/normoxia-induced PAH rat to determine whether altered pyridine nucleotide signaling in the failing heart contributes to 1) increased oxidative stress, 2) changes in metabolic phenotype, 3) autophagy, and 4) the PAH-induced failure. We found that increased reactive oxygen species, metabolic maladaptation, and autophagy contributed to the pathogenesis of right ventricular remodeling and hypertrophy that lead to left ventricular diastolic dysfunction. In addition, arterial elastance increased in PAH rats. Glucose-6-phosphate dehydrogenase is a major source of pyridine molecule (nicotinamide adenine dinucleotide phosphate), which is a substrate for nicotinamide adenine dinucleotide phosphate oxidases in the heart. Dehydroepiandrosterone, a 17-ketosteroid that reduces pulmonary hypertension and right ventricular hypertrophy, inhibited glucose-6-phosphate dehydrogenase, decreased oxidative stress, increased glucose oxidation and acetyl-coA, and reduced autophagy in the hearts of PAH rats. It also decreased arterial stiffness and improved left ventricular diastolic function. These findings demonstrate that pyridine nucleotide signaling, at least partly, mediates PAH-induced diastolic heart failure, and that reduction of glucose-6-phosphate dehydrogenase-derived nicotinamide adenine dinucleotide phosphate is beneficial to improve left ventricle diastolic function. (Hypertension. 2014;64:1266-1274.) • Online Data Supplement

Key Words: dehydroepiandrosterone ■ free radicals ■ heart function tests ■ lung

Heart failure is a major comorbidity and a common cause of the premature death in patients with pulmonary arterial hypertension (PAH). The number of PAH cases is increasing around the world, and, despite recent advances, current medical treatment remains inadequate. Elevated pulmonary arterial pressure increases afterload and decreases right ventricular (RV) function. It is noteworthy, however, that for many years, relatively little effort has been made to study mechanisms of RV hypertrophy (RVH) and failure. As a result, the molecular mechanisms involved in the pathogenesis of RVH remain unknown, and no therapy has yet been identified to prevent or reverse the development of heart failure in PAH.

Left heart failure, an outcome of increased afterload and ischemia or other conditions, is determined by the balance between cell death– and cell survival–promoting mechanisms. Significant progress has been made in understanding the molecular causes of left ventricular (LV) hypertrophy and failure. It is now known that in the failing heart 1) oxidative stress is increased, 2) metabolic phenotype is altered, 3) myocardium is inflamed, 4) the rate of cardiomyocyte apoptotic and autophagic death is augmented, and 5) myocardial fibrosis is induced. Activation of these processes causes myocardial stiffening, dilatation, and dysfunction.

Solid evidence indicates that production of reactive oxygen species (ROS), because of an imbalance between superoxide-generating and antioxidant systems, is elevated in the failing left heart. The nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (Nox-2 and Nox-4) mediate, respectively, neurohumoral and pressure-overload–induced LV hypertrophy. Interestingly, ROS derived from Nox-4...
localized in the mitochondrial matrix also contributes to cardiac myocyte apoptosis and autophagy. In the failing heart, uncoupled nitric oxide synthase—a condition caused when tetrahydrobiopterin is reduced by increased oxidative stress—also generates superoxide anion instead of nitric oxide by donating an electron from NADPH to molecular oxygen. It now seems that Nox- and mitochondria-derived ROS levels go up in the heart before the development of dilated cardiomyopathy. These observations imply that there is a cause–effect relationship between increased myocardial ROS production and heart failure. As in left-sided failure, an association between ROS production and RVH induced by pulmonary hypertension has been observed in humans and rats. Therefore, one goal of this study was to determine whether ROS production goes up in the RV and LV and contributes to the pathogenesis of heart failure in PAH.

NAD(P)H is a cosubstrate for oxidoreductases, such as Nox and nitric oxide synthase in the heart. NAD(P)H/NAD(P)⁺-dependent signaling pathways are emerging as important players in controlling myocardial function, as well as cardiac myocyte survival and death, and more studies are needed to understand the precise role of pyridine nucleotide signaling in causing the failure, as well as the signaling pathways downstream of NADPH-dependent and Nox-derived ROS that reduces the heart function. Although dehydroepiandrosterone is a pleiotropic steroid that can potentially affect multiple biochemical pathways simultaneously in the cell to exert beneficial effects, it is a potent inhibitor of glucose-6-phosphate dehydrogenase (G6PD), which is a major supplier of NADPH in many cells, including the cardiac myocytes.

To our knowledge, dehydroepiandrosterone is the only safe and nontoxic drug available in the market at present that can be used to study the role of pyridine nucleotide signaling in vivo. It reversibly blocks extramitochondrial and mitochondrial G6PD activity and NADPH synthesis, which reduces NADPH supply to Nox in the myocardium, and thereby lowers the myocardial oxidative stress in diabetic and failing hearts. Dehydroepiandrosterone is constitutively secreted from healthy human hearts, where it inhibits the development of cardiac myocyte hypertrophy. In failing hearts, secretion of dehydroepiandrosterone is diminished. Therefore, it is suggested that dehydroepiandrosterone or a metabolite protects the heart by exerting antihypertrophic effects on myocytes. In this regard, dehydroepiandrosterone attenuates L-type Ca²⁺ channel function, which is implicated in activating nuclear factor of activated T-cells, cytoplasmic signaling that causes hypertrophy and failure, and is constitutively secreted from healthy human hearts, where it inhibits the development of cardiac myocyte hypertrophy. In failing hearts, secretion of dehydroepiandrosterone is diminished. Therefore, it is suggested that dehydroepiandrosterone or a metabolite protects the heart by exerting antihypertrophic effects on myocytes.

Figure 1. Reactive oxygen species (ROS) are elevated in right (A) but not in left (B) ventricle of pulmonary arterial hypertension (PAH) rats. Right ventricular ROS are decreased by incubation with tempol (1 mmol/L), a spin trap, ebselen (100 μmol/L), a glutathione (GSH) mimic, apocynin (50 μmol/L), a NADPH oxidase inhibitor, or antimycin (10 μmol/L), a mitochondrial complex III inhibitor, decreased ROS in RV but not in LV. Acpocynin (50 μmol/L) did not
reduce lucigenin chemiluminescence (control, 6155±602 versus apocynin, 6156±501 AU; n=5) signals elicited by xanthine (2.5 mmol/L)+xanthine oxidase (0.06 U/mL).

**Reduced Glutathione Level and Aconitase Activity Are Decreased in the RV but Not in the LV of PAH Rats**

Glutathione detoxifies hydrogen peroxide and hence we measured reduced glutathione levels as a marker of ROS production in RV and LV isolated from PAH and control rats. Reduced glutathione was decreased ($P<0.05$) in RV but not in the LV of PAH rats (Figure 1C).

Aconitase is the first enzyme in the Krebs cycle and it is inhibited by elevated mitochondrial ROS production in the failing hearts$^{24}$; therefore, we measured aconitase activity as a surrogate marker for ROS production in RV and LV from PAH and control rats. Aconitase activity was decreased in the RV but not in the LV of PAH rats (Figure 1D).

**G6PD Activity and ROS Production Are Decreased by Dehydroepiandrosterone Treatment in the RV but Not in the LV of PAH Rats**

Dehydroepiandrosterone, a 17-ketosteroid, is an inhibitor of G6PD. Because G6PD is the major source of NADPH, which fuels ROS production from Noxs in the heart and blood vessels,$^{10,29–31}$ we treated PAH and control rats with dehydroepiandrosterone (1% daily diet) for 3 weeks and then measured G6PD activity and ROS production in RV and LV. Although G6PD activity was not significantly increased either in RV or in LV of PAH when compared with control rats, dehydroepiandrosterone treatment decreased G6PD activity in RV (by 58.1%) and in LV (by 52.6%) of PAH when compared with untreated PAH rats (Figure 2A and 2B), as well as reduced activity and NADPH levels by 53.5% in treated versus nontreated normal hearts. Concomitantly, dehydroepiandrosterone also suppressed ROS production in the RV but not in the LV of PAH when compared with untreated PAH rats (Figure 2C and 2D).

**Pyruvate and Acetyl-CoA Levels Are Increased by Dehydroepiandrosterone Treatment in the RV and in the LV of PAH Rats**

In the normal adult heart, acetyl-CoA is derived from fatty acid and glucose oxidation in the mitochondria. Because cardiac metabolism is altered in failing hearts,$^{32}$ we estimated pyruvate and acetyl-CoA levels in the RV and in the LV of control, PAH, and PAH+dehydroepiandrosterone–treated rats. The levels of pyruvate (by 88±12%) and acetyl-CoA (by 53±18%) were less in the RV than in the LV of control rats. Furthermore, pyruvate (Figure 3A and 3B) and acetyl-CoA (Figure 3C and 3D) were decreased ($P<0.05$) in the LV but not in the RV of PAH rats when compared with control rats. Interestingly, levels of pyruvate and acetyl-CoA were increased by 744% and 730%, respectively, in the RV and by 134% and 178%, respectively, in the LV of PAH rats treated with dehydroepiandrosterone. Tissue lactate:pyruvate ratio, a surrogate measure of cellular NADH-to-NAD$^+$ ratio and levels of NADH that is used by mitochondrial electron transport chain to produce ATP, was increased in the RV (PAH, 0.05±0.0001 and PAH+dehydroepiandrosterone, 0.2±0.08; $P<0.05$; n=5) and in the LV (PAH, 0.06±0.06 and PAH+dehydroepiandrosterone, 0.37±0.08; NS; n=5) by dehydroepiandrosterone treatment when compared with untreated PAH rats.

**Autophagy Is Triggered in the RV but Not in the LV of PAH Rats**

Autophagy plays a role in cell repair and, if uncontrolled, promotes cell death. It is triggered by excess ROS production and by starvation.$^{30,33,34}$ In the heart, autophagy plays a role in hypertrophic remodeling of ventricles, an adaptive response to pressure overload, and also increases cardiac myocyte death and causes heart failure.$^{35}$

Therefore, we performed electron microscopy to determine whether autophagy is triggered in the cardiac tissue of rats by increased ROS production. Electron microscopy of cardiac myocytes in which ROS was elevated showed vacuolar changes partly because of dilated sarcoplasmic cisternae, variably sized and shaped mitochondria, and an autophagic vacuole that contains fragments of organelles when compared with control hearts (Figure 4A and 4B).
We also compared the expression of LC3A/B, an autophagy marker, in the RV and LV from PAH and control rats by Western blot analysis. We detected LC3A/B using antibody from Cell Signaling (Cat # 4108), which predominantly detects type II LC3A/B, and found that the expression of LC3A/B-II increased ($P<0.05$) in the RV but not in the LV of PAH rats when compared with control rats (Figure 4C and 4D).

**Autophagy Is Decreased by Dehydroepiandrosterone in the RV but Not in the LV of PAH Rats**

Because elevated ROS and decreased metabolism trigger autophagy, we determined whether autophagy was reduced in RV and LV by dehydroepiandrosterone. The increased expression of LC3A/B-II was clearly and significantly reduced in the RV (Figure 4C) of PAH+dehydroepiandrosterone–treated when compared with PAH untreated rats. Dehydroepiandrosterone treatment did not alter the normal level of LC3A/B-II in the LV (Figure 4D) of PAH rats.

**Diastolic Heart Failure Is Decreased by Dehydroepiandrosterone in PAH Rats**

Our previous studies show that the RV is severely hypertrophic in PAH rats.36–38 The remodeled RV and septum compress the LV and reduce the volume of the LV cavity. Therefore, we measured LV hemodynamics in PAH+dehydroepiandrosterone–treated, PAH untreated, and control rats. The hemodynamic results are shown in the Table. RV pressure and remodeling were moderately decreased by treating PAH rats with dehydroepiandrosterone.38 Although treatment of PAH rats with dehydroepiandrosterone did not affect dp/dt_{max} (Figure 5A), it normalized dp/dt_{min} when compared with untreated PAH rats (Figure 5B). In addition, dehydroepiandrosterone significantly reduced elevated Tau_g (Figure 5C), end-diastolic pressure (Figure 5D), and arterial elastance ($E_a$=end-systolic pressure/systolic volume=heart rate×resistance; Figure 5E) when compared with untreated PAH rats. Ratio of myocardial elastance ($E_{es}$) to arterial elastance decreased from 0.106 (control) to 0.016 (PAH). Dehydroepiandrosterone treatment increased $E_{es}$ to 0.058 and also increased stroke work (Figure 5F).

![Figure 3. Dehydroepiandrosterone (DHEA) treatment increased pyruvate (A and B) and acetyl-CoA (C and D) levels in the right and left ventricles (RV and LV, respectively) of pulmonary arterial hypertension (PAH) rats. n=5.](http://hyper.ahajournals.org/)

![Figure 4. Electron micrograph of control (A) and increased reactive oxygen species producing (B) right ventricles (RV) showing autophagy in the latter. Asterisk indicates vacuolar changes and white arrow indicates an autophagic vacuole in the pulmonary arterial hypertension (PAH) RV. Expression of LC3A/B-II, an autophagy marker protein, was increased in RV (C) but not in left ventricle (LV; D) of PAH rats. Dehydroepiandrosterone (DHEA) treatment decreased LC3A/B expression in RV but not in LV. n=7 in control and n=5 in PAH and PAH+DHEA groups.](http://hyper.ahajournals.org/)
Cardiac Myocyte L-Type Ca2+ Channel Function and Myocardial Contractility Are Reduced by Dehydroepiandrosterone

The next questions were does dehydroepiandrosterone affect myocardial G6PD activity and NADPH levels and cardiac function? To determine this, we perfused hearts isolated from control, PAH, and PAH + dehydroepiandrosterone rats. Dehydroepiandrosterone (100 μmol/L) also decreased left ventricular developed pressure and dp/dt max (Figure 6C and 6D). Furthermore, dehydroepiandrosterone suppressed L-type Ca2+ channel function (Figure 6E–6L). It decreased currents/current density (Figure 6E and 6F) without affecting steady-state activation (Figure 6G) but by shifting steady-state inactivation curve to the left by 15.4 mV (control, −29.4±0.1 and dehydroepiandrosterone, −44±0.9 mV; Figure 6H). Also, dehydroepiandrosterone decreased currents in a reversible manner (Figure 6I and 6J). Interestingly, the dehydroepiandrosterone-induced reduction of I Ca amplitude, but not steady-state inactivation curve (not shown), was reversed by dialyzing NADPH (100 μmol/L; Figure 6K) and not by dialyzing NADH (100 μmol/L; Figure 6L) into the cardiac myocyte isolated from control rats.

Discussion

The salient findings of our study are 1) ROS production and autophagy were increased in the severely hypertrophied RV of PAH rats, 2) metabolism was not increased in the hypertrophied RVs, 3) PAH rats were in diastolic heart failure, 4) dehydroepiandrosterone treatment attenuated ROS production and autophagy, increased metabolic substrates—pyruvate and acetyl-CoA—and prevented diastolic heart failure, 5) dehydroepiandrosterone decreased G6PD activity in the RV and in the LV of PAH rats, and 6) dehydroepiandrosterone reduced myocardial G6PD activity and NADPH, myocardial contractility, and cardiac myocyte L-type Ca2+ currents.

RV heart hypertrophy and failure, a comorbidity, ensues in a patient with PAH and in animal models of PAH. Heart failure is a major cause of death of patients with PAH. A PAH rat model that mimics clinical features of patients with PAH has elevated RV pressure and develops severe RVH. Interestingly, ROS are also elevated in the severely remodeled RV of these rats. ROS generated by Noxs and mitochondria are elevated in the RV of patients with PAH and rats, and is well known that ROS produced by extramitochondrial Noxs, mitochondrial electron transport chain and Nox-4, and uncoupled eNOS are increased in the failing left heart. Moreover, ROS derived from mitochondrial Nox-4, elicited mainly by pressure overload, and from extramitochondrial Nox-2, elicited by angiotensin-II, contribute to the pathogenesis of LV hypertrophy. Because apocynin, a Nox-2 inhibitor, and antimycin, a mitochondrial complex III inhibitor, decreased ROS levels in the RV, this suggests that ROS were generated by Nox-2 and mitochondrial electron transport chain/Nox-4 activated by elevated neurohumoral factors and pressure overload. Moreover, our findings that ROS production was not upregulated in the LVs of PAH when compared with control rats suggest that increased afterload is, at least partly, essential to increase ROS production in the RV of PAH rats. Although the signaling pathways that cause RV and left heart failure are incompletely understood, this report highlights that RV and LV share common sources of ROS that cause hypertrophy and failure.

NADPH fuels ROS production from Nox in failing hearts and in cardiac myocytes cultured in high glucose. In addition, uncoupled eNOS oxidizes NADPH and increases ROS production in myocardial tissue of failing dog and human hearts. Recently, other authors have proposed a similar mechanism to explain, at least in part, the cardiac oxidative stress in mice with defective glycolytic pathway and aortic constriction. In addition, G6PD-derived NADPH increases reductive stress and plays a significant role in the pathogenesis of protein aggregation cardiomyopathy and heart failure. Therefore, from these observations, it would not be out of context to suggest that increased NADPH cellular content amplifies reductive and oxidative stress in the cardiac myocytes in failing heart. In the adult heart, 60% to 70% of NADPH is produced by extramitochondrial sources increase oxidative stress in the cardiac myocytes in failing heart. In the adult heart, 60% to 70% of NADPH is produced by extramitochondrial and mitochondrial G6PD and ≈30% to 40% of NADPH is derived from mitochondrial malic enzyme and isocitrate dehydrogenase. For this reason, it is speculated that NADPH derived from G6PD-independent sources increase oxidative stress in the LV of G6PD-deficient mice after coronary ligation or transverse aortic constriction. Consistently, although G6PD activity did not increase in the RV or LV (Figure 2), Alzoubi et al reported 4-fold higher NADPH levels in the RV of PAH than that of control rats. This indicates that increased NADPH is derived from either malic enzyme or isocitrate dehydrogenase in the remodeled RVs. Nonetheless, dehydroepiandrosterone treatment lowers NADPH and oxidative stress in the RV of
PAH rats. Because dehydroepiandrosterone decreased G6PD activity in the RV and in the LV from PAH rats (Figure 2), our findings suggest that lowering G6PD-derived NADPH was beneficial to blunt cardiac oxidative stress, which damages myocardium and compromises myocardial function, in the severely hypertrophied RV of PAH rats. However, because dehydroepiandrosterone is a pleiotropic steroid that can potentially affect multiple biochemical pathways simultaneously in the cell to exert beneficial effects, it is plausible that dehydroepiandrosterone reduced cardiac hypertrophy and dysfunction via other pathways in addition to decreasing G6PD/NADPH-dependent ROS generation. Consistent with this speculation, other authors have also reported that G6PD-dependent ROS production plays a role in the pathophysiology of RVH and septal hypertrophy induced by pressure overload. Pyridine nucleotides, NADP(H) and NAD(H), are now emerging as important signaling molecules in controlling cardiac myocyte survival and death, as well as myocardial function. Therefore, the current findings—inhibition of G6PD-reduced hypertrophy and failure in PAH—underscore the notion that the pyridine nucleotide signaling has clinical relevance in cardiovascular medicine.

Along with augmented oxidative stress, cardiac metabolic phenotype is altered in failing hearts. Pyruvate, an end product of glycolysis and lactate oxidation, and acetyl-CoA, a product of pyruvate and fatty acid oxidation, substrates required for energy production were not increased, but the activity of aconitase, the first enzyme that converts citrate to isocitrate in the Krebs cycle, was decreased in the hypertrophic when compared with normal RV. These observations suggest that the metabolic need of the remodeled RV was inadequately compensated. Studies show that cardiac free fatty acid oxidation, the main source of energy in the normal adult heart, is downregulated in failing left hearts, with consequent higher glucose consumption. But glucose is inefficiently catabolized in the glycolytic pathway because of the downregulation of phosphofructokinase in the failing left hearts and is thus unable to meet the energy demand of cardiac myocytes in heart failure. Likewise, the gene profile of proteins involved in regulating fatty acid metabolism and mitochondrial function is downregulated in the dysfunctional RV from PAH rats, and glucose oxidation is decreased in RV from pulmonary artery banding-induced RV overloaded and monocrotaline-induced PH rats. Interestingly, dehydroepiandrosterone treatment enhanced pyruvate and acetyl-CoA levels and increased lactate:pyruvate ratios in the RV and LV of PAH rats. These results suggest that blockade of G6PD activity and ROS production presumably redirected glucose-6-phosphate entry into glycolytic pathway and, at least partly, increased glucose oxidation. Vimercati et al have recently made similar observations in failing dog hearts in which they found that acute inhibition of the pentose phosphate pathway with 6-aminonicotinamide enhanced cardiac glucose oxidation. Therefore, these studies suggest that the inhibition of G6PD may be beneficial to improve metabolism and heart function.

Cardiac myocyte apoptosis or autophagy is triggered by increased oxidative stress and inadequate metabolic compensation. In the heart, autophagy contributes to the pathogenesis of both hypertrophy and failure. Recent studies found that autophagy induced by mitochondrial Nox-4–derived ROS and mitochondrial dysfunction causes remodeling of the RV and LV in pulmonary artery banding and in transverse aortic obstruction.
constriction\textsuperscript{51} models. Consistently, we detected increased autophagy in the hypertrophic RV but not in the LV of PAH rats. Dehydroepiandrosterone, which decreased oxidative stress and increased energy substrates, suppressed increased expression of LC3A/B-II—a marker of autophagy—in the RV of PAH rats. Therefore, we suggest that autophagy, possibly induced by NADPH-dependent increase in oxidative stress, played a role in the pathogenesis of RVH induced by PAH.

Maladaptive RVH is a hallmark of PAH. Although increased afterload, secondary to pulmonary hypertension, is one of the main causes of RVH, elevated neurohumoral factors also contribute to the pathogenesis of heart failure.\textsuperscript{39} In patients with markedly increased RV pressures, LV function is often compromised.\textsuperscript{54} Increased asymmetrical RVH and septal wall thickening compress the LV cavity and cause diastolic heart failure in patients with PAH.\textsuperscript{55} Along these lines, we found that PAH rats were in left heart diastolic failure as indicated by preserved LV ejection fraction, increased dp/dt\textsubscript{max} and isovolumic relaxation time, and elevated filling pressures. Diastolic dysfunction and heart failure are caused by 1) structural changes, 2) inefficient Ca\textsuperscript{2+} sequestration by sarcoplasmic reticulum in diastole, 3) increased fibrosis, and 4) elevated filing pressures because of decreased lusitropy. Interestingly, dehydroepiandrosterone treatment reversed diastolic dysfunction and increased stroke work. In this regard, the human heart synthesizes dehydroepiandrosterone, and dehydroepiandrosterone secretion is reduced in failing human hearts, which leads to the speculation that dehydroepiandrosterone protects the heart by exerting antihypertrophic effects on myocytes.\textsuperscript{71} We attribute the cardioprotective action of dehydroepiandrosterone to its ability to reduce myocardial G6PD-derived NADPH and decrease myocardial NADPH-dependent prohypertrophic signals, such as ROS and I\textsubscript{Ca,L}, which decrease Ca\textsuperscript{2+} entry and left ventricular developed pressure/contractility. Elevated ROS and mitochondrial dysfunction increase intracellular Ca\textsuperscript{2+} and Ca\textsuperscript{2+}-dependent signaling that induces hypertrophy and failure. For example, nuclear factor of activated T-cells, cytoplasmic 3, is activated by increased Ca\textsuperscript{2+} influx through L-type Ca\textsuperscript{2+} channel, and nuclear factor of activated T-cells, cytoplasmic 3–dependent signaling pathway mediates pressure-overload–induced LV failure.\textsuperscript{22} Dehydroepiandrosterone treatment inhibits nuclear factor of activated T-cells, cytoplasmic 3 activation and reduces RVH in PAH rats.\textsuperscript{38} In addition, dehydroepiandrosterone blocks aldosterone-induced neonatal rat cardiac myocyte hypertrophy by reducing T-type Ca\textsuperscript{2+} channel expression and function.\textsuperscript{56} Furthermore, dehydroepiandrosterone, which antagonizes endothelin-1–induced vasoconstriction and relaxes systemic arteries,\textsuperscript{57} may have indirectly reduced diastolic failure by normalizing the increased elastance/stiffening in PAH rats. Arterial stiffness is increased because of the elevation of circulating vasoconstrictors, such as endothelin-1 or serotonin, which rise in PAH. Because G6PD inhibition relaxes pulmonary arteries\textsuperscript{58,59} and dehydroepiandrosterone treatment reduces the severity of pulmonary hypertension in PAH.

Figure 6. Perfusion of isolated rat hearts with dehydroepiandrosterone (DHEA) dose dependently decreased. A, Glucose-6-phosphate dehydrogenase (G6PD) activity and (B) nicotinamide adenine dinucleotide phosphate (NADPH) levels. DHEA (100 μmol/L) attenuated: (C) left ventricular developed pressure (LVDP) and (D) dp/dt\textsubscript{max}. E and F, DHEA (100 μmol/L) decreased cardiac myocyte L-type Ca\textsuperscript{2+} currents (I\textsubscript{Ca,L}). G and H, It shifted the steady-state inactivation curve to the negative potentials and did not affect the steady-state activation. I and J, DHEA-induced inhibition of I\textsubscript{Ca,L} was partially reversible on wash out. K and L, Dialysis of NADPH and not NADH in the pipette solution reversed DHEA-induced suppression of I\textsubscript{Ca,L}. n=5.
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Perspective

Overall, our novel findings demonstrate that 1) ROS production from extramitochondrial and mitochondrial sources are increased in the remodeled RV but not in the LV of PAH rats, 2) dehydroepiandrosterone treatment of PAH rats reduces G6PD activity and NADPH-dependent L-type Ca2+ currents, and 3) dehydroepiandrosterone treatment improves diastolic function in hearts of PAH rats. Therefore, we propose that dehydroepiandrosterone improves function and attenuates hypertrophy by improving metabolism and decreasing ROS production to prevent excess autophagy and by reducing L-type Ca2+ channel function to reduce prohypertrophic transcription factor signaling in the RV of PAH rats. Thus, dehydroepiandrosterone or congeners that suppress NADPH (pyridine nucleotide) signaling may be a beneficial therapy to reduce diastolic heart failure in the PAH syndrome.

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Disclosures

None.

References


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**Novelty and Significance**

**What Is New?**
- Pyridine nucleotide and reactive oxygen species signaling plays a critical role in pulmonary arterial hypertension–induced arterial stiffness and diastolic heart failure.
- Glucose-6-phosphate dehydrogenase inhibitor DHEA treatment decreased pulmonary arterial hypertension–induced arterial stiffness and diastolic heart failure.
- Glucose-6-phosphate dehydrogenase inhibitor DHEA reduced NADPH and reactive oxygen species production in right but not in left heart.
- Glucose-6-phosphate dehydrogenase inhibitor dehydroepiandrosterone improved glucose oxidation and metabolism and prevented autophagy in right but not in left heart.

**What Is Relevant?**
- Heart failure is a hallmark of pulmonary arterial hypertension.
- Rat model of pulmonary arterial hypertension is used.
- Pulmonary arteries undergo severe occlusive remodeling that produces right heart hypertrophy, arterial stiffness, and left heart diastolic failure.

**Summary**
Inhibition of glucose-6-phosphate dehydrogenase suppressed pyridine nucleotide-dependent signaling and reduced myocardial reactive oxygen species and autophagy, improved myocardial glucose metabolism, decreased arterial stiffness, and ameliorated left ventricular diastolic failure.
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Increased reactive oxygen species, metabolic maladaptation and autophagy contribute to pulmonary arterial hypertension-induced ventricular hypertrophy and diastolic heart failure

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Short title: Redox signaling and heart failure

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METHODS:

Animals: All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of South Alabama. PAH was induced in Sprague-Dawley rats (adult males purchased from Harlan Laboratories, Indianapolis, Ind) weighing 180-220 g as described by Abe et al. Briefly, rats were divided into three groups: 1] normal time-matched control group, 2] PAH group, and 3] PAH+DHEA group. Group 1, normal controls; group 2, rats receiving a single subcutaneous injection (20 mg/kg) of SU5416 (Cayman Chemical) on day 1 and then being exposed to 3 wk of normobaric hypoxia (10% O2), followed by re-exposure to normoxia (21% O2) for 5 additional wk (SU/Hx/Nx); and group 3, rats having the same characteristics as in group 2, except for receiving 1% DHEA-containing food (Teklad Custom Research Diet) from weeks 3 to 8 for a total of 5 wk (+DHEA).

Hemodynamic Measurements in Catheterized Rats: Rats were placed on controlled heating pads after they were anesthetized with pentobarbital sodium (30 mg/kg IP). Hemodynamic measurements were performed under normoxic conditions. A microtip pressure-volume catheter (SPR-838, Millar Instruments, Houston, Tx) was inserted into the right carotid artery and advanced into the left ventricle (LV) as described previously. The signals were continuously recorded by the MPVS-300 system with PowerLab/4SP, A/D converter (AD Instruments, Colorado Springs, Colo), and a personal computer. Heart rate, mean systemic arterial pressure, LV systolic/diastolic pressure, LV systolic/diastolic volume, relaxation time and cardiac output were measured. Cardiac index and stroke work are calculated by dividing cardiac output by body weight. At the end of each hemodynamic study, the rat was euthanized by an overdose of pentobarbital sodium, and lungs and hearts were collected for biochemical and histological evaluation and RV/LV+septum (RV/LV+S) weight ratio measurement.

Measurement of Myocardial Function: Myocardial contractile function was determined in isolated hearts as reported previously. Briefly, hearts were isolated and quickly perfused in constant flow mode with Krebs buffer, pH 7.4, containing in mM [NaCl 116.0, NaHCO3 25.0, CaCl2 2.5, MgSO4 1.2, KCl 4.7, KH2PO4 1.2 and glucose 5.5]. The hearts were stabilized for 45-60 minutes, and then perfused with DHEA for 30 minutes after which DHEA was washed out for 60 minutes with normal Krebs solution. Left ventricular developed pressure (LVDP) was recorded by inserting a latex balloon into the left ventricle through the mitral annulus and then inflating the balloon. End diastolic pressure was maintained at 10 mmHg. First derivatives +dP/dt were also determined. Data were collected and analyzed electronically using PowerLab software. Hemodynamic measurements were made continuously and data prior to perfusing the heart with DHEA solution and 30 minutes after DHEA perfusion are reported.

Measurement of Ca2+ Currents in Cardiac Myocytes: Single myocytes were isolated using standard protocols described previously. Freshly isolated cells were dispersed in a small chamber mounted on the stage of an inverted microscope (Nikon, Tokyo, Japan) and superfused with Tyrode solution at room temperature (22-25ºC). Membrane currents were recorded using the patch clamp technique in the whole-cell configuration with Axopatch 1-A amplifiers and pClamp-9 software (Molecular Devices, CA, USA). Whole cell L-type Ca2+ currents (ICa,L) were recorded using previously published solutions and recording conditions. After establishing the whole-cell configuration, membrane capacitance (Cm) was estimated by analyzing the transient charges elicited by a 10 mV pulse from a holding potential of -50 mV. Whole cell currents were filtered at 2 kHz, digitized at 10 kHz, and stored on a computer for off-line analysis. To determine I-V
relationships, pairs of command pulses were applied at 0.2 Hz. After an initial 20-ms ramp pulse from a holding potential of -80 mV up to -40 mV to inactivate the Na⁺ current, square pulses were applied from the holding potential to -40 mV and increased to +50 mV in 10-mV increments. To obtain steady-state inactivation curves for L-type Ca²⁺ channels, Na⁺ was replaced by isomolar tetraethylammonium (TEA), after which I_{Ca,L} were evoked by 500-ms command pulses beginning from a holding potential of -80 mV and then depolarizing to +40 mV in 10 mV increments. The voltage-dependence of the steady-state inactivation was determined from the peak I_{Ca,L} amplitudes during depolarization to a test potential of 10 mV following 2-s prepulses. Normalized peak I_{Ca,L} amplitudes were plotted vs. V and were fitted by a Boltzmann equation: I/I_{max}=1/[1+exp(V_{1/2}-V)/k], where V_{1/2} is mid-voltage of inactivation and k is the slope of the linear portion of the inactivation curve.

After rupturing the cell membrane and establishing a gigaseal, we waited approximately 10 min for currents to stabilize, after which I_{Ca,L} were recorded for up to 10 min after application of drugs. The rundown of I_{Ca,L} was negligibly small before and during the application of drugs; experiments with large rundown were omitted from our analysis.

**Western blot analysis:** Protein was extracted from cells using NP-40 lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% NP-40, 100 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 200 mM pepstatin). Thirty-five μg of sample was loaded and run on SDS-PAGE gels, transferred to nitrocellulose membranes, and subsequently exposed to primary and secondary antibodies and detected by ECL on autoradiography film.

**G6PD Activity:** G6PD activity was measured in the protein extracts by estimating the reduction of NADP⁺ to NADPH. Briefly, enzyme activity was determined in homogenates by measuring the rate of increase of absorbance at 340 nm from the conversion of NADP⁺ to NADPH by G6PD. Substrate concentrations used were glucose-6-phosphate (G6P, 200 μM) and NADP⁺ (100 μM). NADPH fluorescence was detected at 340 nm (Ex) and 460 nm (Em) using an Flx800 microplate fluorescence detector (BioTek Instruments, Winooski, VT).

**NADPH:** Pyridine nucleotides in the myocardial homogenates were estimated by Biovision kit (Milpitas, CA). Briefly, frozen tissue samples were homogenized in extraction medium containing NaOH (0.02 N) and cysteine (0.5 mmol/L) at 0°C. The extracts were then heated at 60°C for 10 min and neutralized with 2 ml of 0.25 M glycylglycine buffer (pH 7.6). The neutralized extracts were centrifuged at 10,000 × g for 10 min, after which the supernatants were passed through 0.45 μm Millipore filters. The filtered solutions were used to measure NADPH levels, which were estimated by determining NADPH fluorescence.

**Superoxide assay:** Protein extracted from heart homogenate was treated with lucigenin (5 µM) in black 96-well plates and chemiluminescence was measured in a microplate reader (BioTek Instruments). Readings were normalized to protein content of corresponding sample.

**Lactate and Pyruvate assays:** Protein was extracted from cells and assays were performed using corresponding kits from Biovision Inc., (Milpitas, CA, USA). Briefly, reaction mixture containing assay buffer, colorimetric probe and enzyme were added to each well in a 96-well plate containing equal volume of protein extract. After incubation
in the dark for 30 min, absorbance was measured at 570 nm in a microplate reader. A standard curve was plotted alongside using lactate/pyruvate provided in the kit. The amount of lactate or pyruvate in samples was determined using corresponding standard curves.

**Acetyl CoA:** Heart acetyl CoA content was estimated by a kit (BioVision Inc.).

**Aconitase Activity:** Heart aconitase activity was determined by a kit assay (OxisResearch, CA, USA).

**GSH Levels:** GSH levels were measured using a GSH reductase-based recycling method using a kit (Cayman Chemical Co., MI, USA).

**Electron Microscopy:** For electron-microscopic examination, hearts were perfusion-fixed under pressure. For fixation, the hearts were perfused with 2.5% glutaraldehyde (buffered in 0.1 M cacodylate adjusted to pH 7.4). The hearts were then kept in the same solution for 1 day and thereafter rinsed with cacodylate buffer with additional 7.5% saccharose. Several blocks from the ventricular free wall were cut and after post-fixing with osmium tetroxide embedded in Epon. Ultra-thin sections were examined electron-microscopically after staining with uranyl acetate and lead citrate.

**Statistical analysis:** Statistical analyses were performed using GraphPad Prizm 5 software. Values are presented as means±SE. All data were analyzed using one-way ANOVA and post-hoc analysis was done by Student’s t-tests. Values of P<0.05 were considered significant.