Severe pulmonary arterial hypertensive rats are tolerant to mild exercise

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Abstract: A frequently used end point of clinical outcomes in patients with pulmonary arterial hypertension (PAH) is the 6-minute walk distance. Furthermore, some data suggest that mild to moderate exercise as an intervention in stable PAH is beneficial. Some of these questions have been recapitulated in the monocrotaline and hypoxia animal models of pulmonary hypertension. However, mild exercise and walk distance as end points have not been rigorously examined in the severe progressive Sugen 5416/hypoxia/normoxia (Su/Hx/Nx) animal model of PAH at each stage of worsening disease. Our hypothesis was that animals that were preselected as runners would have increased walk times and improved right ventricle/left ventricle plus septum (RV/LV+S) ratios, echocardiography, and histology compared with nonexercised Su/Hx/Nx animals. We examined four groups of rats: Su/Hx/Nx sedentary, Su/Hx/Nx exercised, control sedentary, and control exercised. Echocardiography was performed at 5, 8, and 13 weeks to assess right ventricular inner diameter in diastole and left ventricular eccentricity index. We found no difference between exercised and sedentary Su/Hx/Nx rats, and both were worsened compared with controls. Rats were euthanized at 13 weeks, and we found that neither RV/LV+S nor the occurrence of occlusive lesions were influenced by exercise. Most interesting, however, was that despite progressive PAH development, exercised Su/Hx/Nx rats showed no decrease in time or distance for treadmill exercise. In all, our data suggest that, despite severe PAH development, Su/Hx/Nx rats retain the same treadmill exercise capacity as control animals.

Keywords: pulmonary hypertension, exercise, animal models.

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Pulmonary arterial hypertension (PAH) is a progressively fatal syndrome with limited treatment options. The current therapies for PAH do not adequately reduce morbidity or mortality. Exercise is suggested as a preventive for a variety of vascular diseases and has been examined in PAH patients.^{1,2} Fox et al.¹ have found that outpatient pulmonary rehabilitation increased 6-minute walk distance (6MWD) and peak $\dot{V}O_2$ in both PAH and chronic thromboembolic pulmonary hypertension patients. However, in idiopathic PAH there is no increase in 6MWD or peak exercise capacity following a cycling and quadriceps muscle training program. While exercise capacity and endurance were measured in these studies, data on the effects of exercise on the pulmonary vasculature itself are limited.

The effects of exercise have also been examined in several rodent models of PAH with the goal of providing insight into the effects of exercise on the pulmonary circulation. In monocrotaline-injected rats, pulmonary arterial wall thickness and right ventricular capillary density were preserved by exercise, but right ventricular systolic pressure (RVSP) was not improved.³ Exercise training in the hypoxic mouse model improved $\dot{V}O_{2max}$; however, the addition of sildenafil was

required to improve walking distance.³ Furthermore, although RVSP was improved by exercise in hypoxic mice, right ventricular hypertrophy was not corrected. While these data provide insight into whether exercise actually impacts PAH per se, they are inconclusive. This may be somewhat related to the models used and to measurement of these parameters strictly at a final time point.

To examine the effects of exercise over time in this progressively fatal disease, we used the rat Sugen 5416/hypoxia/normoxia (Su/Hx/Nx) model of severe progressive PAH. Recently, the temporal hemodynamic and arteriopathy of this progressive model has been described.⁴ Animals in this model of PAH develop progressive increases in RVSP, right ventricular hypertrophy, and decreased cardiac output.⁴⁻⁷ Su/Hx/Nx rats also develop occlusive pulmonary arterial lesions similar to those observed in PAH patients, including plexiform lesions that are quite evident at 13 weeks.^{4,7} Using this progressive model, we were able to analyze exercise endurance and echocardiography results at multiple time points over the 13-week time course, followed by histological analysis at termination of the study. We hypothesized that exercise would prevent decreases in walk distance and cardiac performance and reduce pulmonary arte-

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rial lesion formation. However, our findings suggest that although both exercised and sedentary rats develop severe PAH, they all retain mild exercise tolerance compared with healthy animals.

METHODS

Animals

All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of South Alabama. All studies in animals were conducted in accordance with the guidelines given in the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*. Adult male Sprague-Dawley rats weighing 160–200 g were injected subcutaneously with Sugen 5416 (20 mg/kg; Cayman Chemical, Ann Arbor, MI) and exposed to normobaric hypoxia (10% O₂) for 3 weeks. They were then returned to normoxia (21% O₂, room air) for 10 additional weeks. Groups studied were (1) control sedentary, (2) control exercised, (3) Su/Hx/Nx sedentary, and (4) Su/Hx/Nx exercised.

Familiarization and exercise testing

Our protocol is an adaptation of that of Fang et al.⁸ Briefly, rats were selected for the exercise study on the basis of willingness to run (with mild electrical stimulation) on a rodent treadmill inclined 5 degrees. Treadmill familiarization took place at a pace of 5 meters per minute for 2 minutes, and the pace was gradually increased to 10 meters per minute for an additional 5 minutes. Rats willing to run were randomly assigned to either control or Su/Hx/Nx groups. Control and Su/Hx/Nx rats were exercised at 5-, 8-, and 13-week time points. The endurance protocol mimicked the familiarization routine for the first 7 minutes. Then the pace was gradually increased 5 meters per minute every 5 minutes thereafter for the duration. The endurance testing was concluded when the animals received a total of three electrical stimulations. Rats that did not

successfully complete the treadmill familiarization period were not exercised during the 13-week period. They served as sedentary comparisons for exercised control and PAH rats (Fig. 1).

Echocardiography

Echocardiography was performed using a Vevo 770 imaging system equipped with a RMV-716 scanhead centered at 17.5 MHz (Visual-Sonics, Toronto, ON). B-mode, M-mode, and Doppler flow imaging was performed at the end of weeks 5, 8, and 13 to evaluate cardiovascular function. Anesthesia was induced using a chamber with 3% isoflurane in O₂. Rats were placed supine on a heated platform and allowed to breath spontaneously. Anesthesia was maintained through a nose cone using isoflurane in O₂ to effect (generally 1.5%–2%). Heart rate and respiration rate were continuously monitored, and adequate anesthesia was determined by lack of a toe-pinch reflex. The right ventricular outflow tract was visualized using a modified parasternal long-axis view. The pulsed-wave Doppler sample volume (length, 1.81 mm) was centrally placed in the lumen of the main pulmonary artery, distal to the pulmonary valve, with the beam oriented parallel to the direction of blood flow. From the Doppler tracings, pulmonary arterial acceleration time (PAAT) was measured as the flow time from start to peak velocity and normalized to total ejection time (duration from start to cessation of flow).⁹ PAAT/pulmonary arterial ejection time (PAAT/PAET) ratios were measured for 10 individual heartbeats and averaged per animal. A midventricular cross section of the heart was visualized using a parasternal short axis view at the level of the papillary muscles. The right ventricular inner diameter in diastole (RVID,d) was measured as the distance from the right ventricular free wall to the interventricular septum, perpendicular to and bisecting the septum.⁹ The left ventricular eccentricity index (LVEI) was measured as the aspect ratio of the left ventricle at end diastole, as defined elsewhere.¹⁰

Figure 1. Schematic of animal groups. Animals were selected on the basis of willingness to run followed by treadmill familiarization. They were then divided into control groups (sedentary and exercised) and Sugen 5416/hypoxia/normoxia (pulmonary arterial hypertension [PAH]) groups (sedentary and exercised).

The index is calculated from one axis diameter parallel to the plane of the interventricular septum divided by a second diameter perpendicular to the first and bisecting the septum. Measurements of RVID,d and LVEI were acquired from at least 10 independent B-mode images at end diastole and averaged per animal.

Histology

Rats were euthanized with an overdose of pentobarbital sodium. Heart and lungs were collected for histological evaluation and to calculate the Fulton index (right ventricle/left ventricle plus septum [RV/LV+S]). Lungs were inflated with 0.5% agarose in 10% neutral buffered formalin at a constant pressure of 20 cm of H₂O before fixation for morphological and histological analysis. The inflated lungs were fixed in 10% neutral buffered formalin overnight. The left lobe was blocked and paraffin embedded. All sections were cut at 5 μ m and stained with hematoxylin and eosin.

Morphological/histological analyses

For analysis of pulmonary arterial occlusions, we counted 200 small pulmonary arteries with an outer diameter of 50 μ m per left lobe cross section from the four groups of rats (one section per rat). Investigators unaware of the experimental group performed the vessel counts. Vessels were assessed for occlusive lesions on hematoxylin and eosin-stained slides and scored as follows: no evidence of neo-intimal formation (open), 50% occlusion or less (partial), and severe (>50%) luminal occlusion (closed). Histological images are provided in Figure S1 (Figs. S1–S3 are available online).

Statistical analysis

Values are means $\pm$ SEM. Analysis of variance was performed with the Bonferroni post hoc test for comparisons among the experimental groups. Differences were considered significant at $P < 0.05$.

RESULTS

Severe PAH animals are tolerant to mild exercise

Using a protocol designed for analysis of mild exercise tolerance in the rodent model of severe PAH (Su/Hx/Nx rats), we analyzed both the duration and the distance of treadmill exercise in control and hypertensive animals. Since PAH is a progressive disease and this model exhibits progressive worsening, we examined both sedentary and PAH animals at early (5 weeks), middle (8 weeks), and late (13 weeks) stages. Each animal was exercised at a similar time during the day for 5 days. We averaged both the time and the distance for each animal over the course of the week. At each time point, there was no significant difference in the duration of exercise tolerated by PAH and control animals (Fig. 2A). In our analysis of the distance traveled during exercise, there was also no significant difference (Fig. 2B).

Interestingly, we did note a significant difference over the time course of the study for both control and Su/Hx/Nx rats. Both sets of animals decreased their exercise time and distance from 5 to 13 weeks. Since animal weight increased over the course of our 13-week study, we analyzed whether increased weight had any bearing on outcome. When both the duration and the distance were stan-

Figure 2. Neither exercise duration nor distance is altered with progression of pulmonary arterial hypertension (PAH) in the Sugen 5416/hypoxia/normoxia (Su/Hx/Nx) model. A, In both control and Su/Hx/Nx PAH rats, we analyzed the time to refusal to run and found that there was no significant difference. B, Distance traveled during the run duration was not different between PAH and control animals ($n = 7$ – 9 animals per group; $P = 0.1115$, not significant).

dardized to animal weight, there were no significant differences between control and PAH groups (Fig. 3).

The above-described data were determined from the average time or distance over a single animal's willingness/ability to run over 5 separate days. Since patient data reported for 6MWD are frequently based on a patient's best reported distance, we then examined whether each animal presented with a "best day." Even when we examined each animal's single best day at each time point, there were no significant differences in tolerance of exercise between control and PAH groups (Fig. 4).

Development of PAH

The severity of PAH development in exercised animals was compared with that in nonexercised sedentary PAH animals and in both exercised and sedentary normoxic controls. RV/LV+S was significantly increased in exercised Su/Hx/Nx animals compared with that in exercised control animals (0.47 ± 0.05 vs. 0.19 ± 0.01 ; $P < 0.05$; Fig. 5A). Sedentary PAH animals also had significantly increased

Figure 3. Standardizing to body weight does not alter exercise duration or distance in both control and Sugen 5416/hypoxia/normoxia (Su/Hx/Nx) rats. A, B, Standardizing the duration of tolerated exercise to the weight of the animals did not influence exercise tolerance. All animals had increased body weight and decreased exercise duration and distance regardless of pulmonary arterial hypertension status ($n = 7-9$ animals per group; $P = 0.1906$, not significant).

RV/LV+S compared with sedentary controls (0.47 ± 0.03 vs. 0.23 ± 0.02 ; $P < 0.05$; Fig. 5A). We also examined right ventricle/body weight ratios and found significant differences between Su/Hx/Nx animals and their respective controls (Fig. 5B). We also examined left ventricle plus septum/body weight ratios, and there were no differences between sedentary and exercised Su/Hx/Nx or control animals (Fig. 5C). Thus, despite the ability to maintain exercise tolerance similar to that in controls, right ventricular hypertrophy in the Su/Hx/Nx PAH rat was not attenuated by mild exercise.

Echocardiography

RVSP, a surrogate of pulmonary arterial pressure, increased by the 5-week time point in Su/Hx/Nx PAH rats and remained ele-

vated over the 13-week time course. Decreased PAAT/PAET correlates with increased pulmonary arterial pressure; thus, to allow for repeated measurements throughout the study of individual animals, we used echocardiography.⁷ Echo measurements were taken at the 5-, 8-, and 13-week time points for cohorts of the sedentary and exercised animals. PAAT was significantly decreased in all Su/Hx/Nx animals, with or without exercise, compared with that in controls at 5, 8, and 13 weeks. Furthermore, there was no statistical difference in PAAT/PAET between the 13-week sedentary Su/Hx/Nx animals and those that were exercised (Fig. 6A). In-

Figure 4. Development of severe pulmonary arterial hypertension (PAH) in the Sugen 5416/hypoxia/normoxia (Su/Hx/Nx) rat does not decrease best-day exercise tolerance. A, B, Selecting the longest duration and distance for each animal as a representative of their best day, we still found no significant difference between control and PAH animals. The only significant difference was an overall decrease in duration and distance from 5 to 13 weeks ($n = 7-9$ animals per group; $P = 0.4553$; asterisks indicate $P = 0.0070$, pound signs indicate $P = 0.0462$).

Figure 5. Sugen 5416/hypoxia/normoxia (Su/Hx/Nx) rats develop severe pulmonary arterial hypertension. A, Su/Hx/Nx rats had increased right ventricle/left ventricle plus septum (RV/LV+S) ratios. B, Su/Hx/Nx animals also had increased right ventricle/body weight (RV/Bw) ratios. C, Su/Hx/Nx animals showed no increase in left ventricle plus septum/body weight (LV+S/Bw) ratios.

increased RVID,d is indicative of elevated RVSP.⁹ RVID,d was increased in Su/Hx/Nx rats at 5, 8, and 13 weeks regardless of exercise or sedentary status (Fig. 6B). Taken together, these data suggest that, despite the observation that Su/Hx/Nx rats maintained their exercise

Figure 6. Both exercised and sedentary Sugen 5416/hypoxia/normoxia (Su/Hx/Nx) rats have increased pulmonary arterial pressure, increased right ventricular pressure, and increased septal flattening indicative of severe pulmonary arterial hypertension (PAH). A, Pulmonary arterial acceleration time/pulmonary arterial ejection time (PAAT/PAET) ratios were not statistically different between sedentary and exercised Su/Hx/Nx rats, indicating increased pulmonary arterial pressure. B, Right ventricular inner diameter in diastole (RVID,d) was increased at 5, 8, and 13 weeks in Su/Hx/Nx rats regardless of exercise status, indicating increased right ventricular pressure. C, Left ventricular eccentricity index (LVEI) was increased in Su/Hx/Nx animals regardless of exercise, indicating septal flattening.

Table 1. Left ventricular eccentricity index (LVEI)

	5 weeks	8 weeks	13 weeks
Sedentary control	1.01 ± 0.023	1.027 ± 0.009	1.048 ± 0.014
Exercised control	1.06 ± 0.014	1.038 ± 0.020	1.048 ± 0.013
Sedentary Su/Hx/Nx	1.87 ± 0.37	1.816 ± 0.067	1.362 ± 0.112
Exercised Su/Hx/Nx	1.78 ± 0.133	1.953 ± 0.105	1.287 ± 0.012

Note: LVEI is the aspect ratio of the left ventricle at end diastole.¹⁰ Su/Hx/Nx: Sugen 5416/hypoxia/normoxia.

tolerance over the entire time course, the severity of PAH was not affected.

Next, we used echocardiography to examine the left ventricular eccentricity index (LVEI), a measure of the ventricular septal flattening observed in PAH.¹⁰ These data were indicative of an increase in right ventricular preload due to elevated right ventricular pressure or volume overload. We found that LVEI at 5 and 8 weeks was significantly increased in Su/Hx/Nx animals compared with controls, regardless of exercise status (Table 1). However, at 13 weeks LVEI in Su/Hx/Nx animals, regardless of exercise status, was not significantly increased compared with that in controls. This may be due to the beginning of decompensation.⁴

Histological analysis of exercised versus sedentary PAH animals

To further examine the severity of PAH in exercised versus sedentary animals, we histologically examined pulmonary vessels for three parameters: open, partial occlusion, and full occlusion. We assessed 200 vessels from one section per animal from the four groups of rats. We specifically examined vessels that were $\leq 50 \mu\text{m}$, on the basis of the work of Toba et al.⁴ that suggested that these are the vessels likely to have the most frequent vascular occlusions. We found that at 13 weeks both sedentary and exercised control animals had no vessel occlusion (Fig. 7). However, both exercised and sedentary Su/Hx/Nx rats had significantly increased partial and severe occlusions (Fig. 7). Therefore, exercise training did not reduce either partial or severe vessel occlusion in Su/Hx/Nx PAH rats.

DISCUSSION

Multiple models of pulmonary hypertension have been previously examined for exercise endurance or the prevention/reversal of pulmonary hypertension by exercise training.^{3,11,12} To our knowledge, however, this is the first study to examine exercise tolerance at various time points in a progressively worsening model of PAH. The major finding of our work is that although Su/Hx/Nx rats develop severe progressive PAH, they are tolerant to mild exercise, and their tolerance is not significantly different from that of healthy rats.

Su/Hx/Nx (PAH) exercised animals had no significant difference in treadmill time or distance at any of the time points examined compared with healthy control animals. This differs from a previous report by Bogaard et al.⁶ that investigated exercise endurance at the 8-week time point and found significantly decreased exercise duration in

Su/Hx/Nx animals. One difference may be in the design of the exercise protocol. Our study was performed at a significantly lower incline and begun at a slower speed. Thus, exhaustion may not have occurred as readily in our animals. In addition, our familiarization was performed prior to selection of animals included in the study, thus eliminating the potential for nonrunners in all groups. Our study did not examine two plausible responses: whether resting or peak $\dot{V}\text{O}_2$ was altered and whether muscle strength or metabolic rate differed between exercised controls and Su/Hx/Nx rats. In all, our study design examines whether progressively worsening PAH alters the response to treadmill exercise, and our findings suggest that tolerance to mild exercise is not influenced significantly by worsening disease.

Our data do fall in line with those of others in different models of pulmonary hypertension. While exercise training in the rat monocrotaline model did decrease pulmonary arterial thickness, it did not prevent right ventricular hypertrophy or improve RVSP.³ An exercise protocol designed for prevention of hypoxic pulmonary hypertension in mice also did not prevent right ventricular hypertrophy.¹¹ Last, exercise training did not improve pulmonary arterial vasoreactivity.¹³ Our data also showed that there is no difference in RV/LV+S, RVID,d, or PAAT/PAET in exercised compared with sedentary Su/Hx/Nx PAH rats, suggesting that there is still right heart dysfunction and potentially severe vasoconstriction regardless of exercise tolerance. While our work does not directly address exercise as an intervention in PAH, our data do support these previous observations.

For classification of patients with PAH and as an outcome measure for disease intervention, the traditional 6MWD is examined. These data are frequently collected on the patients' "best day."¹⁴ Our

Figure 7. Sedentary and exercised Sugen 5416/hypoxia/normoxia (Su/Hx/Nx) rats have increased occlusive vascular lesions. Occlusive vascular lesions in vessels $< 50 \mu\text{m}$ are hallmarks for severe pulmonary arterial hypertension. Untreated animals had no fully occluded vessels, whereas both sedentary and exercised Su/Hx/Nx animals had nearly 50% of their vessels either partially or fully occluded. OD: outer diameter.

data also revealed that although each animal exhibited a best exercise day, these still did not differ significantly between hypertensive and normotensive animals. In addition, exercise intervention improves exercise capacity or 6MWD in patients, and our rat data would support this outcome.^{1,15} While any attempt to interpret animal studies as related to translational work must be done with caution, our study begins to ask the question of whether mild exercise tolerance is an acceptable end point in treatment studies, certainly in rat models.

All parameters measured indicate that Su/Hx/Nx rats have severe progressive PAH. Future studies are needed to address the mechanism of this exercise tolerance, which may include left ventricular compensation or analysis of muscular strength. Our study shows for the first time that mild exercise is easily tolerated in a rat model of severe progressive PAH.

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