CHAPTER 5

Differential effects of exercise training on adrenergic and cholinergic system in stable and progressive pulmonary hypertension

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Abstract

Background: Previously, we demonstrated that exercise training was beneficial in stable pulmonary hypertension (PH), but detrimental in progressive PH. Here we investigate whether these opposite effects of exercise training in stable and progressive PH were related to changes in adrenergic and cholinergic signaling in the right ventricle (RV).

Methods: Two PH-phenotypes were induced by a single injection of monocrotaline of either 40 mg/kg (stable PH; n=18) or 60 mg/kg (progressive PH; n=18). Two weeks after injection, rats were randomized to a sedentary or moderate exercise training protocol. Maximally 4 weeks after, rats were euthanized and the RV was harvested for histological, functional and molecular analyses.

Results: Exercise training induced RV lymphocytes, macrophages and neutrophilic granulocytes infiltration in progressive PH only (all P<0.05). Force measurements of skinned RV cardiomyocytes demonstrated that exercise training increased maximal force in stable and progressive PH (all P<0.05), however passive stiffness was increased only in progressive PH (P<0.05). Myofilament protein phosphorylation revealed that exercise training increased phosphorylation of: myosin binding protein C, troponin T and I (all P<0.05), in stable PH only. Western blot analysis revealed that exercise training increased neuronal norepinephrine reuptake, reduced norepinephrine degradation and increased acetylcholine release and cholinergic receptors expression (both P<0.05), in stable PH only (all P<0.05).

Conclusions: In stable PH, exercise training increased myofilament protein phosphorylation and RV contractility. However, in progressive PH exercise training led to catecholamine-induced myocarditis and increased passive stiffness, and these findings were associated with impaired β-adrenergic receptor signaling, catecholamine reuptake and cholinergic response.
Introduction

Pulmonary arterial hypertension (PH) is a fatal disease characterized by progressive pulmonary vascular remodeling and increased right ventricle (RV) afterload. Previously, we reported opposite effects of exercise training in two PH-phenotypes. We demonstrated that exercise training improved exercise capacity and RV capillary density in rats with stable PH and preserved cardiac output. However in progressive PH, exercise training was detrimental; it not only accelerated disease progression but also resulted in infiltration of leukocytes in the RV.

Normally, the augment in cardiac output during acute exercise results from transient sympathetic nervous system activation, increasing catecholamine levels to raise heart rate and cardiac contractility. However, in PH-patients, impaired RV contractile reserve and RV arterial coupling response upon exercise have been reported. This impaired exercise response could be related to chronic sympathetic nervous system activation leading to downregulation and desensitization of β-adrenergic receptors, which was previously described in PH-patients and animal models of PH. In addition, higher catecholamine levels have a direct cardiotoxic effect and may induce myocarditis. Catecholamine-induced myocarditis may explain the effect of exercise training on RV lymphocytes infiltration in progressive PH.

The parasympathetic nervous system is the natural counterpart of the sympathetic nervous system. Previous studies have suggested that parasympathetic activity may be reduced in PH patients. Recent studies have reported increased parasympathetic activity and cholinergic signaling after exercise training in experimental left heart failure. However, until now the effect of exercise training on cholinergic signaling in PH is unknown.

Therefore in this study, we aim to investigate whether changes in adrenergic and cholinergic signaling could contribute to the opposite effect of exercise training observed in progressive and stable PH. By comparing the effects of exercise training between progressive and stable PH, we were able to demonstrate that impaired β-adrenergic receptor signaling, local catecholamine reuptake and cholinergic response may explain the detrimental effect of exercise training in progressive PH.

Methods

All animal experiments were approved by the Institutional Animal Care and Use Committee of the VU University, Amsterdam, the Netherlands (FYS 06-13).
Experimental pulmonary hypertension
Male Wistar rats were used (n=36; weight, 150 to 175 g; Harlan, Horst, the Netherlands). PH was induced by a single subcutaneous injection of monocrotaline (MCT, Sigma-Aldrich) dissolved in sterile saline. MCT 40 mg/kg was used to induce stable PH and MCT 60 mg/kg was used to induce progressive PH. Two weeks after MCT injection, PH status was confirmed by echocardiography and animals were randomized into sedentary (stable PH-sed or progressive PH-sed; n=9; 5x/week; 1 min; 13.3 m/min; no slope) or moderate exercise training program (stable PH-ex or progressive PH-ex; n=9; 5x/week; 30 min, 13.3 m/min; no slope) as previously described. The training protocol had a maximum duration of 4 weeks or when animals manifest signs of right heart failure (defined as: >5% of body weight reduction, cyanosis, dyspnea and/or lethargy). Rats were euthanized by exsanguination under isoflurane, heart was harvested and stored in liquid nitrogen for further analyses.

RV inflammatory profile
Cardiac cryosections (5 μm) were used to determine cardiac inflammation by immunohistochemistry. Briefly, cryosections were incubated for 60 min with primary antibodies CD45 (lymphocytes), CD68 (macrophages) and MPO (neutrophilic granulocytes) followed by appropriate secondary antibodies, 3,3’-Diaminobenzidine (DAB) and hematoxylin counterstaining to visualize cardiomyocytes membranes.

Protein analyses
RV tissue was treated with trichloroacetic acid to preserve phosphorylation of the myofilament proteins before homogenization for further protein analyses.

Myofilament protein phosphorylation
Phosphorylation of myofilament proteins was determined as previously described. Samples were separated in a gradient gel (Criterion Tris-HCL 4-15%; Biorad). ProQ Diamond Phosphoprotein Stain (Thermo Scientific) was used to determine the amount of protein phosphorylation. Subsequently, gels were fixed, washed, destained and stained with SYPRO Ruby to determine the total amount of protein. The myofilament protein phosphorylation ratio was calculated relative to SYPRO stained myosin binding protein C (MyBP-C) expression to correct for differences in sample loading.

Protein expression by Western blot
Proteins were separated on 1-dimensional gel electrophoresis on 4-12% precast Bis-Tris gels (NuPAGE; Thermo Scientific) and subsequently transferred to nitrocellulose membranes (Hybond ECL Nitrocellulose Membrane; GE Healthcare) using wet transfer. Blots were incubated with the following primary antibodies: norepinephrine
transporter (NET; 1:500; AMT-002; Alomone Labs), monoamine Oxidase A (MAO-A; 1:2000; ab126751; Abcam), choline acetyltransferase (ChAT; 1:1000; AB181023; Abcam), vesicular acetylcholine transporter (VAcHT; 1:500; AB134298; Abcam), alpha-7 nicotinic acetylcholine receptor (α-7nAchR; 1:1000; AB24644; Abcam) and muscarinic acetylcholine type 2 receptor (m2AchR; 1:2000; MA3-044; Thermo Scientific). The protein amount was normalized by the loading control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:50000; G9295, Sigma-Aldrich).

**Isolated cardiomyocyte measurements**

Single cardiomyocytes were obtained by mechanical isolation from the RV free wall, and incubated with 1% of Triton X-100 (Merck Millipore) to permeabilize membranes.16, 18 The permeabilized cardiomyocyte was mounted between a force transducer and a piezo-electric motor, stretched to a sarcomere length of 2.2 μm. Isometric force was measured at various calcium concentrations (ranging from –log[Ca^{2+}] (pCa) 4.5-6.0). To determine passive force (F passive), the cardiomyocyte was transferred to a relaxation solution (pCa 9.0) and shortened for a period of 10 seconds. Active force was calculated by subtracting passive force from the total force at saturating calcium concentrations.16, 18

**Statistical analysis**

Statistical analyses were performed using Prism for Windows (GraphPad 6 Software). Data are presented as mean±SEM, p-values < 0.05 were considered significant. Normality of data was checked and data was log-transformed if it was not normally distributed. To investigate the effects of exercise on different PH phenotypes the two way ANOVA followed by Bonferroni posthoc test was used.

**Results**

**Exercise training induced myocarditis in progressive PH**

To determine whether exercise training could lead to pathological increases in catecholamine-induced myocarditis in progressive PH, levels of lymphocytes, macrophages and neutrophilic granulocytes were assessed in the RV by immunohistochemistry. As previously reported, exercise training dramatically increased RV leukocytes infiltration only in progressive PH (Figure 1A).2 Here we demonstrated that not only lymphocytes levels were increased in progressive PH after exercise training, but also levels of macrophages and neutrophilic granulocytes were increased (Figure 1B-C). This finding suggests that exercise training resulted in a generalized immune response characterized by catecholamine-induced myocarditis only in progressive PH.
Exercise training increased RV levels of lymphocytes (A), macrophages (B) and granulocytes (C) only in progressive PH. *p<0.05, **p<0.01, ***p<0.001, training versus sedentary. Ex=exercise; Sed=sedentary; Stable PH (n=9) and Progressive PH (n=9).

**Exercise training increased passive stiffness in progressive PH**

Functional consequences of exercise training in stable and progressive PH were evaluated by force measurements in skinned RV cardiomyocytes. Maximal force was increased by exercise training in both stable and progressive PH (Figure 2A). Interestingly, the passive stiffness was increased after exercise training solely in progressive PH (Figure 2B). This finding indicates that in progressive PH the increase in maximal force occurs at the expense of the passive stiffness.

Exercise training increased the maximal force in both stable and progressive PH (A), although passive force was increased after exercise only in progressive PH (B). *p<0.05, **p<0.01 training versus sedentary. Ex=exercise; Sed=sedentary; Stable PH (n=5) and Progressive PH (n=5).
Effects of exercise training on myofilament protein phosphorylation

To further investigate whether exercise training induced myocarditis in progressive PH could be attributed to increased sympathetic activity and impaired β-adrenergic receptor signaling, overall sarcomeric protein phosphorylation was determined. Interestingly, exercise training induced opposite phosphorylation changes in stable and progressive PH, which was in particular evident for phosphorylation of the β-adrenergic receptor targets myosin binding protein C and troponin I (Figure 3). No significant difference on desmin and myosin light chain 2 phosphorylation were observed after exercise training (Figure 3E-F). This result suggests that exercise-induced myocarditis in progressive PH could be ascribed to impaired β-adrenergic receptor signaling. Furthermore, it can be proposed that in stable PH, exercise training improved β-adrenergic receptor signaling.

Figure 3: Effects of exercise training on myofilament protein phosphorylation

Exercise training induced divergent effect on myofilament phosphorylation in stable and progressive PH. (A) representative example of proQ diamond staining; (B-D) exercise training significantly increased the phosphorylation of myosin binding protein C (MyBP-C, B), troponin T (TnT, C) and troponin I (TnI, D). However no differences on phosphorylated levels of desmin (E) and myosin light chain 2 (MLC2, F) were observed after training. P-interaction represents the interaction between PH-phenotype and training; *p<0.05, training versus sedentary. SS=stable sedentary; ST= stable trained; PS= progressive sedentary; PT= progressive trained; Ex=exercise; Sed=sedentary; Stable PH (n=9) and Progressive PH (n=9).

Exercise training improved RV catecholamine reuptake in stable PH

To gain more insights into catecholamine-induced myocarditis in progressive PH after exercise training, we assessed catecholamine cycling/catabolism by measuring the RV expression of: norepinephrine transporter (NET), responsible for the reuptake of
extracellular norepinephrine; and monoamine oxidase-A (MAO-A), an enzyme which catalyze amines, such as norepinephrine. In stable PH, exercise training increased NET expression, indicative for neuronal norepinephrine reuptake (Figure 4A) and reduced MAO-A expression, indicative for norepinephrine degradation (Figure 4B), whereas no significant difference on catecholamine cycling/catabolism was observed in progressive PH after training. Taken together, these data indicate that impaired NET and MAO-A response after exercise training may contribute to increase local catecholamine levels in progressive PH.

Figure 4: Effect of exercise training on catecholamine cycling/catabolism

Exercise training increased norepinephrine reuptake (A) and reduced norepinephrine degradation (B) only in stable PH. P-interaction represents the interaction between PH-phenotype and training; **p<0.01, ***p<0.001 training versus sedentary. Ex=exercise; Sed=sedentary; NET=norepinephrine transporter; MAO-A=monoamine oxidase A; SS=stable sedentary; ST= stable trained; PS= progressive sedentary; PT= progressive trained; Stable PH (n=9) and Progressive PH (n=9).

Exercise training increased RV cholinergic signaling in stable PH

To investigate the effects of exercise training on the cholinergic system, protein expression of acetylcholine synthesis (ChAT; choline acetyltransferase), release (VACHT; vesicular acetylcholine transporter) and cholinergic receptors (nicotinic; α-7nAchR and muscarinic; m2AchR) were measured in the RV from stable and progressive PH. Western blot analysis didn’t reveal significant differences in acetylcholine synthesis in both PH-phenotypes (Figure 5A). However, exercise training increased VACHT expression, indicative for acetylcholine release (Figure 5B) and cholinergic receptors expression (Figure 5C-D) only in stable PH. These results suggest an impaired response to cholinergic stimulation upon exercise training in progressive PH rats.
Exercise training did not induce significant differences in acetylcholine synthesis (ChAT) in both PH-phenotypes (A). However, exercise training increased acetylcholine release (VACHT; B) and cholinergic receptors expression (α-7nAchR; C and m2AchR; D) only in stable PH. P-interaction represents the interaction between PH-phenotype and training; *p<0.05, **p<0.01 training versus sedentary. Ex=exercise; Sed=sedentary; ChAT= choline acetyltransferase; VACHT= vesicular acetylcholine transporter; α-7nAchR= alpha-7 nicotinic acetylcholine receptor; m2AchR= muscarinic acetylcholine receptor type 2; SS=stable sedentary; ST= stable trained; PS= progressive sedentary; PT= progressive trained; Stable PH (n=9) and Progressive PH (n=9).

**Discussion**

In this experimental study, we have evaluated the adrenergic and cholinergic system in the RV, as it may explain the divergent effect of exercise training in stable and progressive PH. By combining histological, functional and molecular analyses between stable and progressive PH, we provided evidence that exercise training:

1. Induced RV myocarditis only in progressive PH
2. Increased cardiomyocyte maximal force in both groups, but this occurred at the expense of higher passive stiffness in progressive PH
3. Resulted in opposite β-adrenergic receptor signaling
4. Impaired norepinephrine reuptake in progressive PH
5. Increased acetylcholine release and cholinergic receptors expression in stable PH only

**Opposite β-adrenergic receptor signaling after exercise in PH**

Exercise training is a promising adjunct therapy to reduce sympathetic activity and improve β-adrenergic receptor signaling in animal models\(^\text{19, 20}\) and patients with left heart failure.\(^\text{21, 22}\) However, less is known about the effect of exercise training on the cardiovascular autonomic function in the context of PH. In the present study, we provided evidence that opposite effects on cardiovascular autonomic function may contribute to the divergent effect of exercise training in progressive PH (Figure 6).

Chronic sympathetic nervous system activation leads to increase norepinephrine release, β-adrenergic receptor downregulation\(^\text{6, 7}\), and reduced PKA-mediated phosphorylation.\(^\text{23}\) Increased sympathetic nervous system activity, β-adrenergic receptor downregulation and desensitization have been reported in PH-patients\(^\text{24}\) and animal models of PH.\(^\text{25, 26}\) In addition, we demonstrated that reduced PKA-mediated titin phosphorylation was associated to RV diastolic stiffness in PH-patients.\(^\text{16, 18}\) Here we showed that exercise training leads to opposite myofilament protein phosphorylation, especially in PKA-mediated phosphorylation of cardiac troponin I.

Despite the downregulation of β-adrenergic receptor signaling, higher local norepinephrine production can also contribute to the detrimental effect of exercise training in progressive PH. In the healthy heart, most of the norepinephrine released from sympathetic nerves, 92%, is recaptured by the norepinephrine transporter (NET); around 4%, is removed by the extra-neuronal monoamine transporter into the cardiomyocyte and it will be degraded by the monoamine oxidase A (MAO-A).\(^\text{27}\) Previously, in a PH-rat model impaired NET has been described.\(^\text{26}\) Interestingly, we observed that exercise training induced different responses on norepinephrine cycle/catabolism. Although only expression of NET and MAO-A were measured, we speculate that exercise training resulted in increased norepinephrine levels in the RV in progressive PH-rats, since the norepinephrine released from the sympathetic nerves was not recaptured by the NET, and it was also not degraded by the MAO-A. Higher levels of norepinephrine can induce myocarditis, as previously reported in stress-induced cardiomyopathy\(^\text{28, 29}\), and it could explain the generalized inflammatory response in the RV of progressive PH rats after exercise. Taken together, chronic sympathetic nervous system activity, higher norepinephrine release and impaired β-adrenergic receptor signaling may explain the detrimental effect of exercise training in progressive PH, resulting in RV myocarditis and increased RV diastolic stiffness (Figure 6).
Impaired cholinergic signaling after exercise in progressive PH

Besides the sympathetic nervous system, the parasympathetic nervous system, which is its natural counterpart, may play an important role to maintain the cardiovascular autonomic function in PH. Previous studies have suggested that parasympathetic activity may be reduced in PH-patients, as reduced heart rate recovery after acute exercise was reported in PH-patients. In addition this blunted parasympathetic activity was associated with clinical worsening and exercise capacity. Recent studies in experimental left heart failure have suggested that exercise training improved parasympathetic activity and cholinergic signaling. However, until now the effect of exercise training on cholinergic signaling in PH is unknown.

Acetylcholine is the main neurotransmitter of the parasympathetic/cholinergic system and it is synthesized through choline acetyltransferase (ChAT), an enzyme converting free choline and acetyl-CoA into acetylcholine. The extracellular secretion of acetylcholine is dependent on its packaging into vesicles by the vesicular acetylcholine transporter (VChT). When acetylcholine is released, it can binds to its muscarinic (m2AchR) and nicotinic (α-7nAchR) receptors. In the present study, we have provided evidence that exercise training led to upregulation of VChT, m2AchR and α-7nAchR only in stable PH. Furthermore, we propose that in stable PH the cardiovascular autonomic function was in balance after exercise training, whereas in progressive PH, the sympathetic activity exceeds the parasympathetic system, resulting in an autonomic imbalance in this PH-phenotype (Figure 6B). Therefore, impaired cholinergic signaling after exercise training may also contribute to the detrimental effects of exercise training in progressive PH.
We hypothesized that the different response on β-adrenergic and cholinergic signaling in the RV may contribute to the detrimental effect of exercise training in progressive PH. We observed that exercise training was able to improve norepinephrine reuptake (norepinephrine transporter; NET), and reduced norepinephrine breakdown by MAO-A in stable PH, whereas this was impaired in the progressive PH. In addition, exercise training resulted in opposite PKA-mediated phosphorylation of sarcomeric proteins, improving β-adrenergic receptor signaling only in stable PH. Furthermore, exercise training increased the cholinergic machinery; it increased acetylcholine release (vesicular acetylcholine transporter), nicotinic and muscarinic receptors in the RV of stable PH. We propose that the cardiovascular autonomic function was in balance after exercise training in stable PH, whereas in progressive PH, a predominant increase in the sympathetic activity may contribute to the detrimental effects of exercise training.

Clinical perspective

Exercise training is an adjunct therapy able to improve quality of life and exercise capacity of PH-patients. However, it remains to be established whether exercise training is beneficial for all PH-patients or should be limited to patients with stable PH. In our present study, we provide evidence that impaired β-adrenergic and cholinergic signaling may contribute to the detrimental effects of exercise training in progressive PH. It could therefore be speculated that autonomic function could be used to distinguish the response to exercise training. Future clinical studies should further investigate how to identify the PH-patients who will benefit from exercise training.
Conclusions

In stable PH, exercise training increased β-adrenergic receptor signaling and RV contractility, whereas the detrimental effect of exercise training in progressive PH could be associated with impaired β-adrenergic receptor signaling, catecholamine reuptake and altered cholinergic signaling, coinciding with myocarditis and RV diastolic stiffness.

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