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Differential Efects of Exercise Training on Mofilament Phosphorylation and Function in Stable and Progressive Pulmonary Hypertension

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ABSTRACT

Background – We have previously demonstrated opposite effects of exercise training in stable and progressive pulmonary hypertension (PH). Here we investigated cellular changes which may underlie the opposite effects of exercise training by focusing on right ventricular myofilament protein phosphorylation and function.

Methods and results – Histological analyses revealed exercise training in progressive PH induced massive leukocyte ($P_{interaction}<0.01$), granulocyte ($P_{interaction}<0.05$) and macrophage ($P_{interaction}<0.05$) infiltration only in the right ventricle. By ProQ diamond analyses, we observed that phosphorylation of the myofilament proteins myosin binding protein C ($P_{interaction}<0.01$), troponin T ($P_{interaction}<0.05$) and troponin I ($P_{interaction}<0.05$) was increased by exercise in stable PH and reduced in progressive PH. In progressive PH, reduced protein phosphorylation was associated with increased protein phosphatase 1 (PP1) expression. Myofilament Ca^{2+}-sensitivity was increased in progressive PH ($p<0.001$) and reduced ($p<0.01$) in stable PH. Exercise training increased maximal force in both stable ($p<0.05$) and progressive PH ($P<0.01$), while passive force was significantly elevated upon exercise in progressive PH ($p_{interaction}<0.05$).

Conclusions – The beneficial effect of exercise training in stable PH was associated with increased myofilament protein phosphorylation and RV contractility, whereas in progressive PH exercise training induced severe myocarditis, increased PP1 expression, reduced myofilament protein phosphorylation and resulted in increased passive stiffness.

INTRODUCTION

Pulmonary Arterial Hypertension (PH) is a fatal disease characterized by excessive remodeling of the pulmonary arterioles. Previously, two phenotypes of PH have been described in a rat model: stable PH with preserved cardiac function and progressive PH with right heart failure. In this rat model, we observed opposite effects of exercise training for stable and progressive PH.[1] In stable PH, exercise training was beneficial as it improved exercise capacity and increased right ventricular (RV) capillary density.[1] In contrast in progressive PH, exercise training accelerated the progression to right heart failure (RHF) which was accompanied by massive infiltration of lymphocytes in the right ventricle.[1]

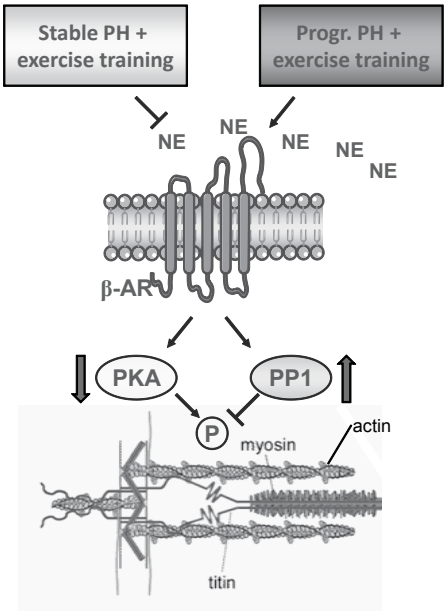
Stable and progressive PH could be distinguished hemodynamically, as in stable PH cardiac output was preserved, whereas in progressive PH cardiac output declined over time.[1] A decrease in cardiac output is a primary trigger of neurohumoral activation.[2] Normally, when cardiac output has to increase in response to exercise, catecholamine levels are temporarily increased to raise heart rate and cardiac contractility.[3] An increase in cardiomyocyte contractility is obtained via phosphorylation of sarcomeric proteins and proteins involved in calcium handling by protein kinase A (PKA), the downstream kinase of the β-adrenergic receptor.[4-6] Cardiomyocyte performance upon β-adrenergic receptor stimulation is further enhanced by blockade of protein phosphatase 1 (PP1).[7] In both right and left heart failure, levels of catecholamines are chronically elevated to maintain cardiac output.[8,9] However, this pathological chronic increase in catecholamine levels results in down-regulation and desensitization of the β-adrenergic receptor signaling pathway and as a consequence reduced PKA-mediated protein phosphorylation.[10] In addition, increased PP1 expression and activity have been reported in left heart failure.[11] This increase in PP1 seems to be associated with chronic catecholamine stimulation of the β-adrenergic receptors, as PP1 content was normalized by chronic β-blocker therapy in an infarct pig model.[12] Thus apart from reduced PKA-mediated phosphorylation, chronically increased catecholamine levels may further reduce protein phosphorylation by an increase in PP1. Previous studies demonstrated that in a well established rat model of PH-induced right heart failure, plasma norepinephrine levels are increased and β-adrenergic receptor density of the right ventricle is decreased.[13] Hence, the decline in cardiac output in progressive PH may involve reduced phosphorylation of proteins as a consequence of chronic β-adrenergic receptor stimulation.

Apart from the detrimental effects on intracellular signaling, the pathological high levels of catecholamines have direct cardiotoxic effects and can even induce myocarditis.[14,15] The high levels of lymphocytes in progressive PH and further increase with exercise indicate that detrimental effects of high catecholamine levels may be further exacerbated by exercise. In contrast, in stable PH with preserved cardiac output, exercise may exert beneficial effects via improvement of β-adrenergic receptor signaling, as beneficial effects of exercise training in an infarct mouse model were mainly attributed to improvement of β-adrenergic signaling

Figure 4.1 Hypothesis

Schematical overview of the mechanisms (i.e. divergent effect on adrenergic activity) which may underlie opposite effects of exercise training in stable and progressive PH. As adrenergic activity is difficult to measure directly, we focused on the consequences of adrenergic activity and analysed the presence of stress-induced myocarditis, myofilament protein phosphorylation and cardiomyocyte function. As depicted in the figure, high levels of catecholamines (due to elevated adrenergic activity) can result in receptor downregulation, reduced PKA-activity and as a consequence hypophosphorylation of the myofilaments (consisting of actin, myosin and titin). Alternatively, increased PP1 expression/activity may reduce myofilament protein phosphorylation.

Abbreviations: NE, norepinephrine; β-AR, beta-adrenergic receptor; PKA, protein kinase A; PP1, protein phosphatase 1; P, phosphorylation.



and correction of myofilament function.[16] On the basis of previous studies, we hypothesize that exercise training induces opposite alterations in myofilament protein phosphorylation and function in stable and progressive PH (Figure 1).

To establish that exercise training aggravates myocarditis in progressive PH we have extended our microscopic analyses with the quantification of macrophage and granulocyte infiltration. To reveal alterations in myofilament phosphorylation, phosphorylation status of the myofilament proteins and PP1 expression were determined. Cardiomyocyte function was assessed by force measurements in single permeabilized cardiomyocytes. Our findings indicate that exercise training in progressive PH induced severe myocarditis, myofilament hypophosphorylation and increased diastolic stiffness, whereas in stable PH protein phosphorylation was elevated and cardiomyocyte contractility was improved.

METHODS

All experiments were approved by the Institutional Animal Care and Use Committee at the VU University.

Experimental pulmonary hypertension

Male wistar rats were used (36 in total; weight 150-175 g; Harlan, Horst, The Netherlands). PH was induced by a single injection of monocrotaline (MCT, Sigma-Aldrich, Zwijndrecht, The Netherlands) dissolved in sterile saline. MCT 40 mg/kg was used to induce stable PH with a preserved cardiac output (n=18) and progressive PH developing right heart failure (n=18) was induced by a dose of 60 mg/kg MCT. After 2 weeks of MCT-treatment all rats had developed PH, and rats were randomized to sedentary (5x /week; 1 min; 13.3m/min; no slope) or exercise training (5x /week; 30 min; 13.3m/min; no slope) as previously described.[1] Rats were trained for a maximum of 4 weeks or shorter when rats developed clinical signs of heart failure earlier (defined as: >5% loss of body mass /day and/or respiratory distress, cyanosis, lethargy). After euthanization by exsanguination, hearts were harvested and stored in liquid nitrogen for further analyses.

Inflammation

Analysis of cardiac inflammation was performed by immunohistochemistry. Cardiac cryosections of 5 µm were stained for 60 minutes with the primary antibodies CD45 (lymphocytes), CD68 (macrophages) and MPO (granulocytes).[17] Primary antibodies were visualized with appropriate secondary antibodies and 3,3'-Diaminobenzidine (DAB) as well as hematoxylin counterstaining to visualize cardiomyocytes membranes.

Protein analyses

All right ventricular samples were treated with trichloroacetic acid, to preserve phosphorylation of the myofilament proteins, before samples were homogenized for further protein analyses.[18]

Myofilament protein phosphorylation status

Phosphorylation of myofilament proteins was determined as described before.[18] Samples were separated on a gradient gel (Criterion Tric-HCL 4% to 15% gel, BioRad) and proteins were stained for one hour with ProQ Diamond Phosphoprotein Stain (Molecular Probes). Fixation, washing, and destaining were performed according the manufacturers guidelines. Subsequently, gels were stained with SYPRO Ruby staining for determination of total myofilament protein levels. The phosphorylation status of myofilament proteins was expressed relative to SYPRO stained myosin binding protein C (MyBP-C) expression to correct for differences in sample loading.[19]

Protein Phosphatase 1 expression

Proteins were separated by 1-dimensional gel electrophoresis on 4% to 15% precast Tris HCL gels (Bio-Rad laboratories, Hercules, Calif) and subsequently transferred to nitrocellulose paper by semi-dry blotting. Blots were incubated with a primary antibody against protein phosphatase 1 (sc-7482, mouse monoclonal antibody, Santa Cruz Biotechnology). Primary antibody binding was visualized by incubation of the appropriate secondary antibody and enhanced chemiluminescence (Amersham, GE Heakthcare, Chalfont St. Giles, UK).[12]

Isolated cardiomyocyte measurements

Single cardiomyocytes were obtained via mechanical isolation from the RV free wall, and incubated with Triton X-100 (0.5%) to remove all membranes.[20] The permealized cardiomyocyte was subsequently mounted between a force transducer and a piezoelectric 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 cell was transferred to relaxation solution (pCa 9.0) and shortened for a period of 10 sec. Active force was calculated by subtracting passive force from total force ($F_{active} = F_{total} - F_{passive}$) at saturating Ca^{2+} concentrations.[21]

Statistical analyses

All analyses were performed in a blinded fashion. All data were verified for normal distribution. Data are presented as mean±SEM and a p-value < 0.05 was considered significant.

Changes in force-calcium relationship was tested with two way analysis of variance for repeated measurements; Interaction between training-status and calcium concentration was tested to evaluate if the force developed at various calcium concentrations was different between training and sedentary rats. For all further analysis, two way ANOVA of variance was used; Interaction between PH-status and training-status was tested and Bonferroni post-hoc tests were performed (training vs. sedentary in the two experimental groups). All reported p-values of post-hoc comparisons are Bonferroni corrected (SPSS 16.0 for Windows, SPSS, Chicago IL).

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

RESULTS

Inflammatory cell infiltration in the RV in progressive PH by exercise training

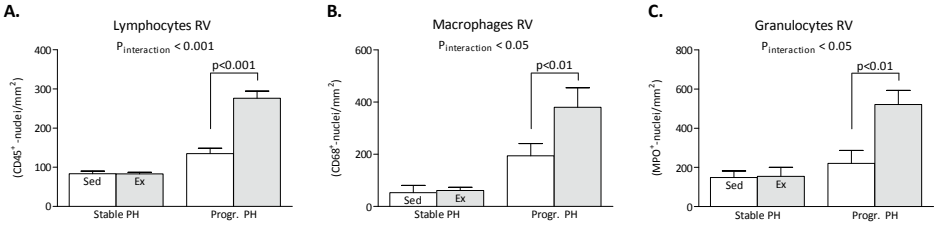
To examine whether catecholamine levels were elevated to pathological levels, we assessed whether catecholamine-induced myocarditis was present. As previously shown, exercise training dramatically increased RV leukocyte infiltration in progressive PH only.[1] Here, we

Figure 4.2 Exercise training induces RV inflammation only in progressive PH

Exercise in progressive PH induces RV inflammation, characterized by increased levels of lymphocytes (A), macrophages (B) and granulocytes (C). An inflammatory response to exercise was absent in stable PH.

Data presented as mean ±SEM. *** p<0.001; ** p<0.01 training vs. sedentary. $P_{interaction}$ represents the interaction between type of PH and training.

Abbreviations: RV, right ventricle; PH, pulmonary hypertension; $CD45^+$ -nuclei, nuclei stained positive for lymphocytes; $CD68^+$ -nuclei, nuclei stained positive for macrophages; MPO^+ -nuclei, nuclei stained positive for granulocytes; S, sedentary; T, training.



demonstrate that also levels of macrophages and granulocytes are increased by exercise training in progressive PH (both $p_{interaction}$<0.05; Figure 2). This indicates that the previously found lymphocyte infiltration is part of a generalized immune response characterized by a catecholamine-induced myocarditis. In contrast, exercise training did not evoke an immune response in stable PH. Moreover, inflammation was specifically observed in the right ventricle and was absent in the left ventricle.

Effects of exercise training on myofilament protein phosphorylation

By ProQ-diamond staining, we were able to assess myofilament protein phosphorylation in stable and progressive PH (Figure 3A). All phosphorylation data are expressed relative to protein expression of MyBP-C, which expression was similar in all groups.

Note the divergent phosphorylation by exercise training of all myofilament proteins in stable and progressive PH. However, only for MyBP-C ($p_{interaction}$<0.01), troponin T (TnT; $p_{interaction}$<0.05) and troponin I (cTnI, $p_{interaction}$<0.05) this divergent phosphorylation reached statistical significance. In stable PH exercise training significantly increased phosphorylation of one of the main PKA myofilament target proteins, cTnI. As phosphorylation of all proteins was reduced by exercise training in progressive PH, we assessed phosphatase expression by western blot analyses. As shown in Figure 4, PP1 expression was significantly increased (Sedentary: 2.8 ±0.7 vs. Training: 7.1 ±2.2 a.u.; p<0.01) solely in rats with progressive PH and exercise training (Stable PH, Sedentary: 1.8±0.6 vs. Training: 2.5 ±0.7 a.u.).

Exercise training increased passive stiffness in progressive PH

To evaluate the functional consequences of altered myofilament protein phosphorylation, we performed functional cardiomyocyte measurements (n=5 per group; 3 cells per rat). A representative skinned cardiomyocyte and a force recording are demonstrated in Figure 5A,B. Maximal force development at pCa 4.5 was increased by exercise training in both stable (Sedentary: 44.3 ±2.7 vs. Training: 54.9 ±2.7 kN/m^2; p<0.05) and progressive PH (Sedentary:

Figure 4.3 Phosphorylation of the myofilament proteins

Divergent effect of exercise training on myofilament phosphorylation in stable and progressive PH. A) Typical example of proQ diamond gel. B-F) Phosphorylation of myosin binding protein C (MyBP-C; B), Desmin (C), Troponin T (TnT; D), Troponin I (cTnI; E) and myosin light chain 2 (MLC2; F). Data presented as mean ±SEM. * $p<0.05$ training vs. sedentary. $P_{interaction}$ represents the interaction between type of PH and training. Abbreviations: sPH-S, stable PH sedentary; sPH-T, stable PH training; pPH-S, progressive PH sedentary; pPH-T, progressive PH training; S, sedentary; T, training.

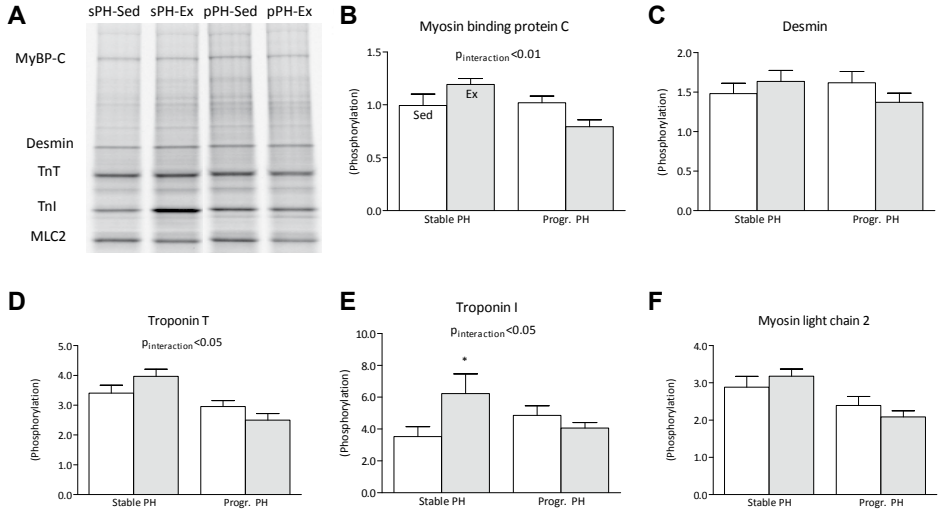


Figure 4.4 Exercise training increased protein phosphatase expression in progressive PH

Exercise training increased protein phosphatase 1 expression only in progressive PH.

Data presented as mean ±SEM. * $p<0.05$ training vs. sedentary. Abbreviations: S, sedentary; T, training.

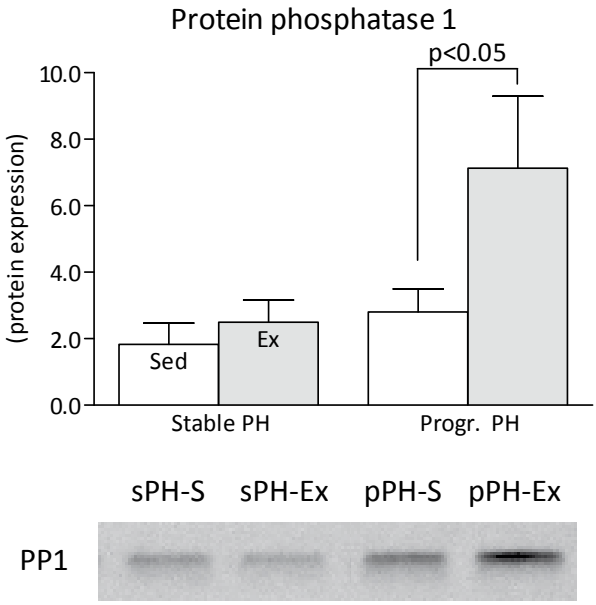
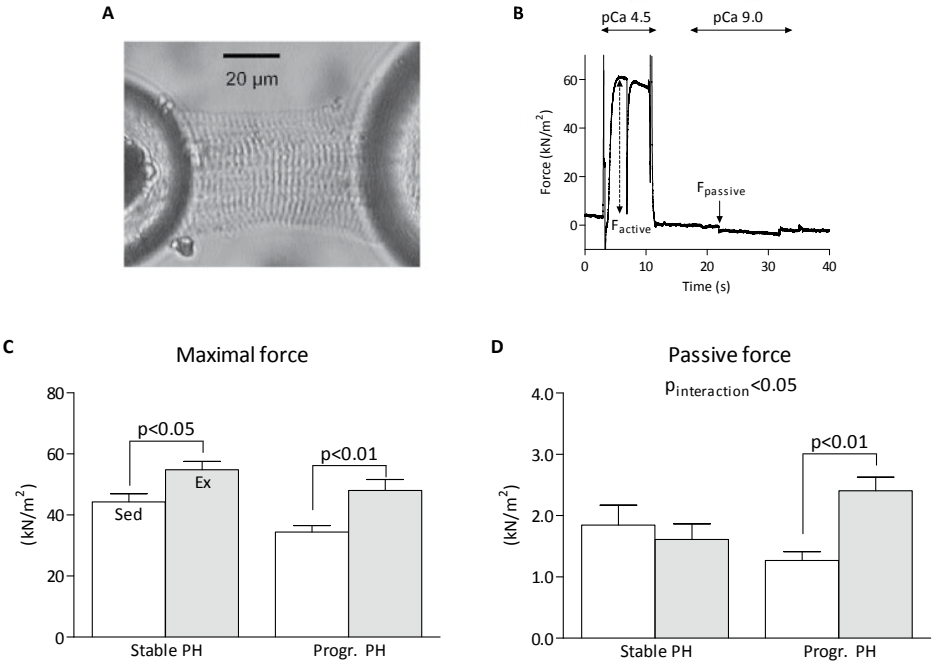


Figure 4.5 Effect exercise training on myofilament function in stable and progressive PH

A) Single skinned cardiomyocyte at a sacromere length of 2.2 µm. B) Typical recording of myofilament function measurements. The cell was quickly shortened by 30% and immediately restretched to its original length at saturating calcium concentrations (pCa 4.5) to determine total force (F_{total}). Subsequently, the cell was transferred to relaxation solution (pCa 9.0) and shortened for a period of 10 sec. to determine passive force ($F_{passive}$). Active force was calculated by subtracting passive force from total force ($F_{active} = F_{total} - F_{passive}$). C) Exercise training increased maximal force in both stable and progressive PH. D) Passive force was significantly increased after exercise training in progressive PH, whereas exercise had no effect in stable PH.

Data are presented as mean ±SEM. * p<0.05, ** p<0.01 training vs. sedentary. Abbreviations: F_{total}, total force; $F_{passive}$, passive force; F_{active}, active force.



34.4 ±2.1 vs. Training: 48.0 ±2.6 kN/m^2; p<0.01). Interestingly, the divergent effect of exercise training was again observed in passive force ($p_{interaction}$<0.05). Exercise training had no effect on passive stiffness in stable PH (Sedentary: 1.9 ±0.3 vs. Training: 1.6 ±0.3), whereas exercise training almost doubled passive force in progressive PH (Sedentary: 1.3 ±0.1 vs. Training 2.4 ±0.2 kN/m^2; p<0.01).

Figure 6 shows the average force-calcium relationships of stable PH (A) and progressive PH (B). After exercise training in stable PH, the force-calcium curve is shifted to the right (p<0.001), indicating that more calcium is needed to generate the same force as sedentary stable PH rats. In contrast, in progressive PH the curve is shifted to the left (p<0.01), indicating that less calcium is needed to generate the same force as sedentary progressive PH rats. This is also demonstrated in Figure 6C; where exercise training tended to reduce myofilament Ca^{2+}-sensitivity (pCa_{50}) in stable PH, whereas pCa_{50} tended to be increased after exercise training in progressive PH ($p_{interaction}$ = 0.067).

Figure 4.6 Effect of exercise training on calcium sensitivity in stable and progressive PH
Exercise training tends to induce an opposite effect on calcium sensitivity in stable and progressive PH. A) Force-calcium (pCa) relationship in stable PH. Dotted line represents training animals. Note that exercise training in stable PH shifted the curve to the right, indicating more calcium is needed for the same force generation (i.e. reduced myofilament Ca^{2+}-sensitivity). B) Force-calcium relationship in progressive PH. Dotted line represents training animals. Note that exercise training in progressive PH, shifted the curve to the left, indicating that less calcium is needed for the same force generation in comparison with sedentary animals (i.e. increased myofilament Ca^{2+}-sensitivity). C) There is a trend that exercise training has an opposite effect on myofilament Ca^{2+}-sensitivity (pCa_{50}) in stable and progressive PH.
All data is presented as mean ±SEM. Abbreviations: pCa, negative logarithm of calcium concentrations, high pCa = low calcium concentration and low pCa = high calcium concentration; pCa_{50}, represents the calcium concentration at which 50% of the active force is generated and is a measure of calcium sensitivity of the cardiomyocytes.

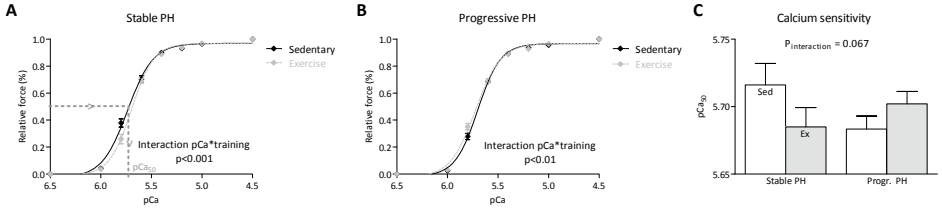
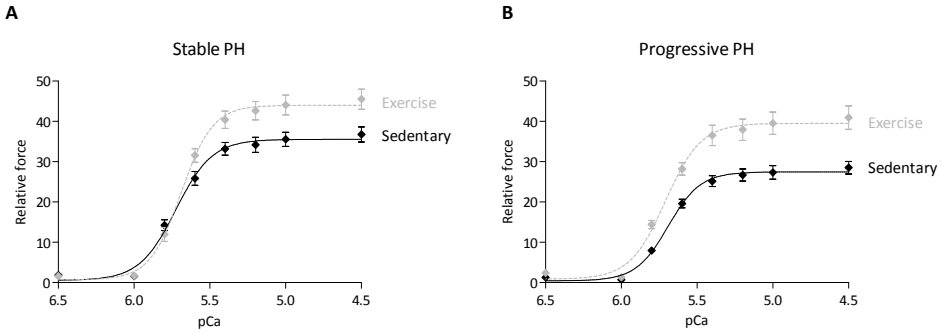


Figure 4.7 Exercise training increased systolic function in progressive PH at the expense of diastolic function
Exercise training in both stable and progressive PH increased maximal force. However, this increase in systolic function in only in progressive PH accompanied by an increase in passive force of the cardiomyocytes. A) Absolute force-calcium relation in stable PH in sedentary (solid line) and training (dotted line). Note the increase in force only at high calcium concentrations. B) Absolute force-calcium relation in progressive PH in sedentary (solid line) and training (dotted line). Note the increase in force at both low and high calcium concentrations. All data presented as mean ±SEM. Abbreviations: pCa, negative logarithm of calcium concentrations, high pCa = low calcium concentration and low pCa = high calcium concentration.



It can be depicted from Figure 7A,B that the observed increase in active force in progressive PH with exercise training is at the expense of the passive force, whereas in stable PH only active force improves without changing passive force.

DISCUSSION

In this study we have demonstrated that in stable and progressive PH exercise training induced divergent alterations in:

1. Myofilament protein phosphorylation

2. Passive stiffness
3. Myofilament Ca^{2+}-sensitivity

These findings indicate that in manifest right heart failure myofilament phosphorylation and function are affected. Future research should evaluate if these myofilament alterations can serve as therapeutic target to reduce or even prevent the development of overt right heart failure.

Divergent myofilament protein phosphorylation and function

Exercise training is often used as therapeutic strategy to reduce cardiovascular risk factors in patients with left heart failure.[22] The beneficial effects of exercise training on left ventricular function are mainly ascribed to alterations in β-adrenergic receptor signaling and Ca^{2+}-handling.[16,23,24] In line with these studies we could demonstrate increased myofilament phosphorylation, in particular of the PKA target protein cTnI, and improved active force development in stable PH, which had a favorable response to exercise training as we have previously reported.[1]

Also in progressive PH myofilament active force generating capacity was improved by exercise training. However, the increased active force concided with an increase in passive stiffness.

The increase in passive stiffness in progressive PH, may involve hypophosphorylation of titin. Titin is the largest protein known in physiology and spans the half sarcomeric distance from Z-disk to M-line. Titin is the main determinant of cardiomyocyte passive stiffness and it consists of three elements: the serially linked immunoglobulin-like domains; the N2B-element and the PEVK-element.[25] Recently it has been reported that phosphorylation of titin by PKA and protein kinase C α (PKCα) can have opposite effects on cardiomyocyte stiffness. PKA-mediated phosphorylation of the N2B element of titin *reduces* cardiomyocyte stiffness and contributes to ventricular relaxation. Alternatively, PKCα increase cardiomyocyte stiffness via phosphorylation of the PEVK-element.[26,27] Apart from changes in kinase activity, the observed increase in PP1 expression may reduce titin phosphorylation. Phosphorylation of myofilament proteins analysed in the present study was lowest in the trained progressive PH group (Figure 3). Hence PP1 induced hypophosphorylation of titin may underly the increased cardiomyocyte stiffness upon exercise in progressive PH. Further research should focus on the role of titin phosphorylation in the development of diastolic dysfunction in progressive PH, as this may be used as therapeutic target for the prevention of right heart failure.

Possible mechanisms

Exercise training induced catecholamine-induced myocarditis only in progressive PH rats. Previous research in monocrotaline-treated animals has indicated that sympathetic nervous system (SNS) activity is elevated in rats developing right heart failure.[28,29] Catecholamines have direct cardiotoxic effects[30] and are associated with elevated pro-inflammatory cytokine

expression (TNF-α, IL-1β and IL-6).[15] Bouts of exercise will further increase SNS-activity, and the consequent catecholamine overload might have been detrimental for the right ventricle in progressive PH.

However this is not in line with the finding that exercise training is beneficial in patients with chronic left heart failure, even in the more severe patients who are known to have increased SNS-activity.[31] Moreover, beneficial effects of exercise training have also been reported for a genetic mouse model with sympathetic hyperactivity (α_{2A}/α_{2C} adrenoreceptor knock out mouse).[32] We therefore speculate that wall stress plays an additional role in progressive PH. We previously have demonstrated that hypertrophy is comparable between stable and progressive PH, whereas RV afterload is significantly higher in progressive PH.[1] As a consequence, the wall stress of the right ventricular cardiomyocytes is severely elevated in progressive PH. Moreover, wall stress was speculated to even further increase during exercise due to an increase in afterload as was previously reported in PH-patients.[33] Elevated wall stress can increase catecholamine levels and up-regulate pro-inflammatory cytokine expression. Sun *et al* observed significant up-regulation of TNFα levels already after 10 minutes of stretch in cardiomyocytes.[34] Wall stress as determinant of the response to exercise is further illustrated by the preliminary findings of van Deel *et al*, demonstrating that exercise training is only beneficial in mice with chronic heart failure induced by myocardial infarction and not in mice with transverse aortic constriction (TAC).[35]

Taken together these studies suggest that both SNS overactivity in combination with increased wall stress could have attributed to the detrimental effects of exercise training in progressive PH.

Conclusions and clinical implications

The present study demonstrates that the beneficial effects of exercise training in stable PH was associated with improved β-adrenergic receptor signaling, evident from increased phosphorylation of the PKA target protein cTnI, and increased RV contractility, whereas in progressive PH exercise training induced severe myocarditis, reduced myofilament phosphorylation and resulted in increased passive stiffness. Increased catecholamine levels as a consequence of SNS overactivity together with elevated wall stress of the right ventricle are likely to contribute to these detrimental effects of exercise training in progressive PH. As beta-blocker therapy is a well-known strategy to prevent catecholamine overspill in left heart failure, our data support its clinical application for treatment of right heart failure and should be further evaluated in progressive PH.

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