REDUCED FORCE OF DIAPHRAGM MUSCLE FIBERS IN PATIENTS WITH CHRONIC THROMBOEMBOLIC PULMONARY HYPERTENSION

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COMPETING INTERESTS

No, there are no competing interests

RUNNING TITLE: Diaphragm muscle weakness in CTEPH-patients
ABSTRACT (244 words)

RATIONALE - Patients with pulmonary hypertension (PH) suffer from inspiratory muscle weakness. However, the pathophysiology of inspiratory muscle dysfunction in PH is unknown. We hypothesized that weakness of the diaphragm, the main inspiratory muscle, is an important contributor to inspiratory muscle dysfunction in PH-patients.

OBJECTIVES - To combine ex vivo diaphragm muscle fiber contractility measurements with measures of in vivo inspiratory muscle function in chronic thromboembolic pulmonary hypertension (CTEPH) patients.

METHODS - To assess diaphragm muscle contractility, function was studied in vivo by maximum inspiratory pressure (MIP), and ex vivo in diaphragm biopsies of the same CTEPH-patients (N=13) obtained during pulmonary endarterectomy. Patients undergoing elective lung surgery served as controls (N=15). Muscle fiber cross sectional area (CSA) was determined in cryosections and contractility in permeabilized muscle fibers.

RESULTS - Diaphragm muscle fiber CSA was not significantly different between control and CTEPH-patients in both slow-twitch and fast-twitch fibers. Maximal force generating capacity was significantly lower in slow-twitch muscle fibers of CTEPH-patients, while no difference was observed in fast-twitch muscle fibers. The maximal force of diaphragm muscle fibers correlated significantly with MIP. The calcium sensitivity of force generation was significantly reduced in fast-twitch muscle fibers of CTEPH-patients, resulting in a ~40% reduction of submaximal force generation. The fast skeletal troponin activator CK-2066260 (5µM) restored submaximal force generation to levels exceeding those observed in control subjects.

CONCLUSIONS - Diaphragm muscle fiber contractility is hampered in CTEPH-patients and contributes to the reduced function of the inspiratory muscles in CTEPH-patients.

Keywords: Myocyte physiology, contractile proteins, respiratory capacity, dyspnea
INTRODUCTION

Pulmonary hypertension (PH) is a progressive disease and despite improvements in disease-targeted therapies, PH-patients remain symptomatic and have a reduced survival (21). Symptoms include limited exercise capacity and dyspnea (38), which are not only related to cardiac dysfunction, but also to dysfunction of peripheral (3, 4, 25, 31) and inspiratory muscles (1, 22, 28, 29, 32).

Several studies have found a marked reduction of maximal inspiratory pressures in PH-patients compared with control subjects (22, 32). The underlying cause of the inspiratory muscle weakness is unknown, but might include contractile dysfunction of the inspiratory muscles, in particular the diaphragm (1, 28). For instance, in chronic heart failure (CHF) and chronic obstructive pulmonary disease (COPD), individual muscle fibers isolated from diaphragm biopsy specimens showed contractile weakness (14, 24, 34). Furthermore, diaphragm muscle fiber size is reduced in animal models of PH as well as in biopsies of end-stage PH-patients, and the force generating capacity of individual diaphragm muscle fibers is reduced in animal models of PH (1, 28, 29). However, it is currently unknown whether these changes are also present in PH-patients.

Therefore, in the present study, we measured in vivo inspiratory muscle function and ex vivo diaphragm muscle fiber contractility within the same patients. To determine ex vivo diaphragm muscle fiber contractility, biopsies are indispensable. For this reason, we focused on patients with PH due to operable chronic thromboembolisms (CTEPH). Patients included all underwent a pulmonary endarterectomy, during which the diaphragm becomes readily accessible and biopsies could be obtained. We hypothesized that the contractile force and the size of individual fibers is reduced in CTEPH-patients and that these changes at the fiber level can, at least partly, explain the inspiratory muscle weakness. The contractile force was assessed by measuring maximal force generating capacity, cross-bridge cycling kinetics and calcium sensitivity of force generation in permeabilized diaphragm muscle fibers. In addition, to augment diaphragm fiber
contractile strength in CTEPH-patients, we tested the ability of a novel, small molecule drug CK-2066260 to improve the calcium sensitivity of force.
METHODS

Subjects and respiratory muscle function testing

Muscle biopsies of the mid-costal diaphragm were obtained from CTEPH-patients (CTEPH, N=13) during pulmonary endarterectomy and from patients without PH during elective thoracotomy for resection of a small pulmonary tumor (CTRL, N=15, note that comparable patients served as controls in many previous studies (18, 19, 23, 34)). In addition, muscle biopsies of either the pectoralis major or rectus abdominis were obtained from the CTEPH patients (Non-DIA, N=13). One part of the fresh biopsy was frozen in liquid nitrogen and stored at -80°C for later analysis. A second part was placed in a relaxing/glycerol (50/50) solution containing high concentrations of protease inhibitors (DTT 0.5 mM, leupeptin 0.04 mM, E64 0.01 mM) and placed overnight on a roller band at 4°C. Subsequently, the relax/glycerol solution was refreshed and the biopsy was stored at -20°C till further use. Exclusion criteria included: weight loss of > 10% in the 6 months prior to surgery (to exclude cachexia), primary lung disease (including COPD), congenital myopathies or dystrophies, neurodegenerative disorders and chronic use of corticosteroids (>7.5mg/day) or other drugs that are known to affect muscle strength.

Spirometry and maximal inspiratory and expiratory pressures were assessed in the CTEPH-patients (N=9) 1-2 days prior to surgery as described previously (11). In brief, patients were sitting in an upright position and breathed through a flanged mouthpiece. Maximal inspiratory pressure (MIP) was determined from a forceful inspiratory effort against a shutter, initiated at functional residual capacity. MIP is a negative pressure, but is expressed as a positive value. Maximal expiratory pressure (MEP) was measured during maximal expiratory effort at total lung capacity. MIP and MEP were determined from the best of 3-5 consecutive maneuvers; average standard deviation between the consecutive maneuvers was 0.9kPa.

This study was approved by the local ethics committee and written informed consent was obtained from each subject.
**Histology**

To determine fiber cross sectional area (CSA) and fiber type distribution, 5 μm cryosections were cut and incubated for 60 minutes with primary antibody against fast-twitch muscle fibers (MY31, 1:35, Sigma-Aldrich, Zwijndrecht, the Netherlands) in 0.5% bovine serum albumin (BSA) in phosphate buffer saline (PBS), followed by an appropriate secondary antibody and wheat germ agglutinin (WGA) staining of the cell membranes (Molecular Probers, Eugene, Oregon, USA). Following each incubation cryosections were washed 3 times for 3 minutes with 0.1% Tween in PBS. Finally, the sections were embedded in Vector Shield without DAPI and closed with cover glass-slides. Image acquisition was performed with SlideBook imaging. Image J was used to semi automatically quantify the images. Analyses were included when a minimum of 30 cells per fiber type per patient was measured.

Note that, when snap-freezing the biopsy it is possible that the muscle shortens which may influence the CSA. We were unable to measure muscle fiber sarcomere length in these frozen samples and can thus not correct for potential differences in fiber length. However, the biopsy was pinned on a cork before it was frozen in liquid nitrogen to prevent shortening of the muscle. Previous analysis in our lab revealed that with this method the sarcomere length in the frozen tissue is quite consistent, also across different patient groups (20).

**Single muscle fiber contractile measurements**

Single muscle fibers (~1.0 mm in length) were isolated from the diaphragm and non-diaphragm muscle tissue stored at -20°C using micro forceps. The fiber was attached between two aluminum foiled clips and incubated in 1% Triton X-100 relaxing solution for 10 minutes to permeabilize the membranes. For the composition of the solutions used, see below (29).

A single fiber was mounted on a single-fiber apparatus on top of an inverted microscope. The fiber was placed between a force transducer (model 403A, Aurora Scientific, Aurora, Ontario, Canada) and a servomotor (315C, Aurora Scientific). Fibers that appeared damage
during microscopic examination were excluded from the study. All measurements were performed at 20°C (29, 34, 35).

The composition of the relaxing solution (with a total ionic strength of 180 mM) consisted of 5.89 mM Na₂ATP, 6.48 mM MgCl₂, 40.76 mM K-propionate, 100 mM BES, 6.97 mM EGTA and 14.5 mM CrP with sufficient KOH to adjust the pH to 7.1. Activating solutions ranging from a [Ca²⁺] of 0.1 to 32 μM (maximal activation) were obtained by appropriate mixing of relaxing and activating solution. The composition of the 'jump' solution was similar to the relaxing solution but with an EGTA concentration of 0.1 mM (29, 35).

Single fiber contractile experiments were performed as described previously (29). In brief, while the fiber was in relaxing solution, sarcomere length was set at 2.5 µm using a fast Fourier transformation on a region of interest on the real-time camera image. The fiber was activated shortly by placing it in activating solution ([Ca²⁺] 32 μM), sarcomere length was checked afterwards and adjusted when necessary. Muscle fiber length, width and depth were measured using the live camera image. The cross section area (CSA) was calculated assuming that the fiber cross section is ellipsoidal. All contractile experiments were performed at a sarcomere length of 2.5 µm and are expressed as tension (force per CSA).

To determine the tension-[Ca²⁺] relationship, the fiber was placed for 1 minute in jump solution followed by activating solutions with incremental Ca²⁺ concentrations ranging from 0.1 till 32 μM, and the isometric force generation was recorded. Force values at submaximal [Ca²⁺] were normalized to the maximal force obtained at 32 μM [Ca²⁺] to determine Ca²⁺-sensitivity of the fiber expressed as EC₅₀, i.e. the [Ca²⁺] at which 50% of maximal force is reached. The EC₅₀ was determined by fitting a modified Hill equation through the data points.

The effect of the fast-troponin activator CK-2066260 was tested in diaphragm fibers from a subset of CTEPH-patients (N=6) and controls (N=6). A concentration of 5 μM of CK-2066260 was used based on previous studies with the same compound and similar tissue (18). Fibers were measured in solutions with 5 μM CK-2066260 followed with solutions containing vehicle
(1% DMSO), or first measured in vehicle and subsequently measured with solutions containing 5 μM CK-2066260.

The rate constant of force redevelopment ($\kappa_{tr}$) was measured in activating solution by rapidly releasing the fiber by 30% of its original length, followed by a quick restretch to its original length. The $\kappa_{tr}$ was determined by fitting a double exponential through the force redevelopment curve (note that only the fast rate constant is reported as this is considered to reflect cross-bridge cycling kinetics (6, 15)).

Following the $\kappa_{tr}$ protocol, active stiffness was determined by imposing small length perturbations of 0.3, 0.6 and 0.9 % on the fiber resulting in a quick force response (Fig. 1). The tension change ($\Delta T$) was plotted as a function of the length change ($\Delta L$). Active stiffness was derived from the slope of the fitted line and is a measure to estimate the number of cycling cross-bridges. The ratio of maximal tension and active stiffness reflects the force generated per cross-bridge (Fig. 1).

**MHC isoform composition and MHC content**

At the end of the single fiber contractile protocol, the fibers were detached from the force transducer and servomotor and the fiber was placed in 25 μL of SDS sample buffer. MHC isoform composition was determined by SDS-PAGE as described previously (10, 29). In brief, the samples were denaturated by boiling for 2 minutes. A homogenate of control diaphragm muscle was run on each gel for comparison of migration patterns of the MHC isoform and, from known amounts of purified rabbit MHC (M-3889; Sigma) run on every gel, a standard curve was constructed to determine MHC content in the single fibers. The gels were silver stained and scanned with an image densitometer, and optical densities of the electrophoretic bands were quantified. Total MHC content of the fiber was determined (in 25μL SDS buffer) based on the standard curve. MHC concentration was calculated by dividing total MHC content by muscle fiber volume. We discriminate only between slow-twitch and fast-twitch fibers. Note that the fast-twitch fibers (137 fibers) consisted mainly of type 2A fibers (109), with 4 type 2X fibers and
24 type 2A/2X fibers. Fibers that co-expressed both slow-twitch and fast-twitch MHC isoforms were excluded from further analysis (cut off value of 75% of one type).

Statistical analysis

Statistical analysis were performed using Graphpad Prism 5 for Windows (Graphpad Software Inc, San Diego, CA) and SPSS version 20 (SPSS Inc. Chicago, Illinois). Normal distribution was tested and if necessary logarithmic transformation was applied. If the data was normally distributed multilevel analysis to correct for non-independence of successive measurements per patient (MLwiN, 2.02.3; Centre for Multilevel Modelling, Bristol, UK) was used (12, 26, 27, 29). If data could not be analyzed with multilevel analysis an independent student-t test or Mann Withney U test was used on the averages per patient. A two-way repeated measure ANOVA was used to analyze differences in force-[Ca^{2+}] relation with a Bonferroni post-test. The contractile parameters were tested with a paired t-test for diaphragm and non-diaphragm muscle of the CTEPH-patients. A multilevel approach was not chosen because of unequal pairs in different fiber type groups, which results in loss of data. A p-value of <0.05 was considered significant.
RESULTS

Subjects' Characteristics

Patients' characteristics, pulmonary function and respiratory muscle strength are shown in table 1. No differences between CTEPH and control patients were observed with regard to gender, age, body mass index, and pulmonary function.

Histology

The CSA of fast-twitch and slow-twitch diaphragm fibers was assessed in control subjects (N=15) and CTEPH-patients (N=11), see Fig.2A. No significant difference in CSA was observed between groups in both slow-twitch and fast-twitch muscle fibers (Fig.2B). To assess whether fiber type proportions differed in the diaphragm of CTEPH-patients, we determined the percentage of total muscle fibers that consisted of slow-twitch fibers. No significant difference was observed between groups (CTRL vs. CTEPH 55±4 vs. 55±2 [%], p=0.87).

Single muscle fiber contractile measurements

A total of 280 individual fibers of CTEPH-patients (N=13) and controls (N=15) were manually isolated from the diaphragm biopsies and used for contractile measurements. The distribution of the fiber types is provided in table 2. Fiber types 2A, 2X and 2A/2X were pooled and are referred to as fast-twitch muscle fibers. Because of the low number, fibers that co-expressed type MHC slow and 2A were excluded from further analysis. Due to technical difficulties, not all parameters could be measured in all fibers. The number of patients per parameter is indicated in the figures. In case a multilevel analysis was used, the number of the individual muscle fibers is indicated above the bars.

Maximal tension – The maximal force-generating capacity - normalized to CSA (i.e. tension) - was determined in single permeabilized diaphragm muscle fibers of CTEPH-patients and controls. Maximal tension of slow-twitch muscle fibers was significantly lower in
CTEPH-patients than in controls (Fig.3A). No difference in maximal tension was observed in fast-twitch muscle fibers.

**Cross-bridge cycling kinetics** - To evaluate the underlying cause of the reduction in maximal tension we studied the cross-bridge cycling kinetics. The active tension generated by permeabilized muscle fibers is determined by: 1) the number of available cross-bridges; 2) the fraction of strongly bound cross-bridges ($\alpha_{fs}$); and 3) the force generated per cross-bridge (5, 10). A reduction in maximal tension should be accompanied by a change in one or more of these three determinants.

First, we measured active stiffness by imposing small length changes on the fiber during maximal activation (for details see Fig.1). The force change during these length perturbations is caused by stretch of the bound cross-bridges (not by passive-elastic structures in the muscle fibers), and therefore active stiffness provides an estimate of the number of attached cross-bridges during activation. A reduction in active stiffness was observed in slow-twitch muscle fibers of CTEPH-patients (Fig.3B) while no change was observed in fast-twitch muscle fibers. This finding suggests that the number of attached cross-bridges is reduced in slow-twitch muscle fibers of CTEPH-patients. We also measured the rate of force redevelopment ($\kappa_{tr}$) during maximal activation. No significant difference in $\kappa_{tr}$ was observed between groups in both slow-twitch (CTRL vs. CTEPH: 5.09±0.11 vs. 5.03±0.13[s⁻¹]) and fast-twitch (CTRL vs. CTEPH: 14.24±0.65 vs. 14.14±076[s⁻¹]) muscle fibers. This suggests that the reduced number of attached cross-bridges is not a result of a reduced fraction of attached cross-bridges ($\alpha_{fs}$) (note that caution is warranted: to conclusively establish that $\alpha_{fs}$ is unaltered, stiffness measurements during rigor conditions or measurements of the rate of cross-bridge detachment would be necessary). Finally, we estimated the force generated per cross-bridge by calculating the tension/stiffness ratio. No significant difference was observed between groups (Fig.3C), indicating that the reduction in maximal tension was proportional to the reduction in active stiffness.
Thus, the data from these mechanical measurements suggest that the reduction in maximal tension in slow-twitch diaphragm fibers of CTEPH-patients is caused by a reduction in the number of bound cross-bridges. Next, we studied whether this reduction in the number of bound cross-bridges in slow-twitch diaphragm fibers was a result of a reduced concentration of myosin, the main contractile protein. Indeed, as shown in Figure 4, the myosin heavy chain concentration in slow-twitch diaphragm fibers of CTEPH-patients was significantly reduced compared to that in diaphragm fibers of control subjects (note that these biochemical assays were performed in the same slow-twitch diaphragm fibers as were used for the mechanical measurements).

**Calcium sensitivity of force** - During normal inspiration, the diaphragm is not maximally activated, but is activated at submaximal firing rates. Therefore, we measured the force response at submaximal [Ca$^{2+}$] and determined the Ca$^{2+}$-sensitivity of force. As shown in Fig. 5A, no shift in the force-[Ca$^{2+}$] curve was observed in slow-twitch muscle fibers of CTEPH-patients, indicating unaltered Ca$^{2+}$-sensitivity of force. In fast-twitch muscle fibers of CTEPH-patients a right-ward shift of the force-[Ca$^{2+}$] relation was observed (Fig.5B), indicating reduced Ca$^{2+}$-sensitivity of force. The [Ca$^{2+}$] at which 50% of maximal tension is reached (EC$_{50}$) was determined in all fibers, and a significant increase in EC$_{50}$ was found in fast-twitch muscle fibers (CTRL vs. CTEPH: 0.61±0.09 vs. 0.76±0.18 [μM], p<0.05), whereas no change was observed in slow-twitch muscle fibers (CTRL vs. CTEPH: 0.82±0.10 vs. 0.84±0.07 [μM]). As a result, the tension (i.e. force per CSA) of fast-twitch muscle fibers was significantly reduced at [Ca$^{2+}$] of 0.63μM; a concentration close to the EC$_{50}$ (Fig. 5C).

Next, we tested the ability of the fast skeletal troponin activator CK-2066260 to improve contractility at submaximal [Ca$^{2+}$] in fast-twitch muscle fibers in a subset of CTEPH-patients (N=6) and controls (N=6; note that these experiments were not powered to detect differences in EC$_{50}$ between fibers of CTEPH (DMSO) and control (DMSO) subjects, which explains the less pronounced leftward shift of the force-Ca$^{2+}$ relation in Fig.5D than in Fig.5B). Previous work from our group showed that 5μM of CK-2066260 yields a near-maximal effect (18); therefore,
this concentration was used in the present study. As CK-2066260 specifically targets fast
troponin C (36), no effect on the Ca\textsuperscript{2+}-sensitivity of force in slow-twitch muscle was observed
(Fig. 5E). However, in fast-twitch muscle fibers - the fiber type that showed a reduced
Ca\textsuperscript{2+}-sensitivity of force in CTEPH-patients - 5μM CK-2066260 significantly increased the
Ca\textsuperscript{2+}-sensitivity of force in both control and CTEPH-patients (EC\textsubscript{50} CTRL: DMSO vs. CK 0.72±0.03
vs. 0.26±0.07[μM], p<0.05. EC\textsubscript{50} CTEPH: DMSO vs. CK 0.79±0.05 vs. 0.28±0.05 [μM], p<0.05) (Fig.
5D). As a result, tension at [Ca\textsuperscript{2+}] of 0.63μM was significantly increased in fast-twitch muscle
fibers of CTEPH-patients during exposure to CK-2066260 (Fig. 5E). That CK-2066260 had a
comparable effect on force in diseased (i.e., CTEPH) fibers compared to healthy (i.e., control
subjects’) fibers is in line with previous studies on troponin activators (36).

\textbf{In vivo inspiratory muscle function}

The average MIP, measured in CTEPH-patients (6.2±2.4kPa, N=9) 1-2 day prior to pulmonary
derarterectomy, was comparable to previously reported values in PH-patients (Table 1) (22,
32), and was lower than normal values (2). We sought to find correlations between MIP and
diaphragm muscle fiber contractility and size. For these correlations we pooled both fiber types,
as MIP is a reflection of the contractile strength of all diaphragm fibers together. Maximal
diaphragm muscle fiber force showed a strong correlation with MIP (Fig. 6). Both diaphragm
muscle fiber CSA and maximal tension (i.e. force normalized to CSA) contribute to maximal force,
but neither significantly correlated with MIP (P > 0.05 in both instances). The maximal force of
diaphragm muscle fibers did not significantly correlate with 6 minute walking test (r\textsuperscript{2}=0.24,
p=0.18). Finally, hemodynamic parameters (e.g. mPAP, CO and PVR) did not correlate with MIP
or with the maximal force of diaphragm fibers (P > 0.05 in all cases).

\textbf{Non-diaphragm muscle function in CTEPH-patients}

Diaphragm muscle biopsies were obtained during PEA, during which the patient is placed on
cardiopulmonary bypass and cooled to a core temperature of ~18°C (39). As this procedure
alone might affect muscle function, we also obtained a biopsy of the pectoralis major (N=9) or rectus abdominus muscle (N=4) in the same patient. A comparison between the contractility of fibers of the diaphragm and from these non-diaphragm muscles is shown in figure 7. In slow-twitch fibers of CTEPH patients, maximal tension of non-diaphragm fibers was significantly higher compared to that of diaphragm fibers of the same patients (and, importantly, comparable to that of diaphragm fibers of control subjects, Fig.3A). In fast-twitch fibers of CTEPH patients, maximal tension of non-diaphragm fibers was comparable to that of diaphragm fibers (and to that of diaphragm fibers of control subjects, Fig. 3A). These findings suggest that surgery by itself does not greatly affect skeletal muscles in general. This is further supported by the correlation of MIP, measured pre-operatively, with the contractility of individual diaphragm muscle fibers obtained during pulmonary endarterectomy (Fig. 6).
DISCUSSION

The major findings of this study are that:

(1) the maximal force generating capacity of slow-twitch diaphragm muscle fibers is reduced in CTEPH-patients.

(2) the calcium sensitivity of force is reduced in fast-twitch diaphragm muscle fibers of CTEPH-patients, a reduction which was restored by the fast skeletal troponin activator CK-2066260.

(3) diaphragm muscle fiber contractility correlates with MIP, suggesting that weakness of diaphragm muscle fibers contributes to the reduced contractile strength of the inspiratory muscles in CTEPH-patients.

Reduced contractility of diaphragm muscle fibers correlates with inspiratory muscle weakness in CTEPH-patients

Several studies have reported on inspiratory muscle weakness in PH-patients (22, 32). The average MIP (6.2kPa) measured in CTEPH-patients in the present study was comparable to that previously reported in PH-patients (~6kPa), and is lower than the MIP of control subjects (~8kPa) (22, 32). Thus, the cohort of CTEPH-patients suffered from inspiratory muscle weakness.

Thus far, the pathophysiology that underlies this weakness has not been completely understood. Previous studies in animal models with PH demonstrated a reduction in diaphragm muscle fiber size and sarcomeric dysfunction (1, 29). Similar changes have also been observed in a pilot study on biopsies of PH-patients (28). However, based on the low number of patients and the absence of fiber typing in that study a definite conclusion could not be drawn. Importantly, in the present study we combined measurements of in vivo and ex vivo inspiratory muscle contractility in individual CTEPH-patients.
We observed no reduction in the CSA of diaphragm muscle fibers of CTEPH-patients, in contrast to a significant reduction in CSA reported previously in PH-patients (28). This discrepancy might be a consequence of the current study not being designed to detect changes in fiber CSA (it was powered on muscle fiber contractility), and therefore the statistical power to detect changes in fiber CSA might not have been sufficient. Alternatively, the discrepancy with previous work might be explained by the fact that in the previous study end-stage PH-patients were studied. Hemodynamics in end-stage PH-patients are much more compromised than those of the CTEPH-patients studied here, likely resulting in more pronounced inspiratory muscle weakness (9). The CTEPH-patients studied here were selected for PEA, an advanced surgical intervention that is performed only on stable patients (thus cachectic patients, who are likely to exhibit diaphragm fiber atrophy, were excluded). In non-failing PH-rats, also no reduction in diaphragm muscle fiber CSA was observed (28). Therefore, we propose that reductions in diaphragm fiber size only occur in end-stage disease, whereas changes in contractility occur already at earlier disease-stages.

To evaluate diaphragm muscle fiber contractility, biopsies are indispensable. These biopsies are obtained from the belly of the diaphragm. Hence, normal excitation-contraction coupling is disrupted and we therefore permeabilized the muscle fibers. In permeabilized muscle fibers the membranous structures are made permeable while leaving the sarcomeres intact. By exposing these fibers to exogenous calcium, fiber contractility was studied. These experiments revealed that diaphragm muscle fiber contractile function was impaired in CTEPH-patients. Maximal tension was reduced in slow-twitch muscle fibers, and the calcium sensitivity of force was reduced in fast-twitch fibers. As a result, submaximal tension was reduced by ~40% in fast-twitch muscle fibers of CTEPH-patients (Fig.5C, note that this was also reduced in slow-twitch fibers, but that the reduction did not reach significance), and by ~25% when both slow-twitch and fast-twitch muscle fibers were combined. This is an important finding, as in vivo the diaphragm is typically activated at submaximal firing rates, which
generates submaximal force generation. Thus, reduced contractility of diaphragm fibers might substantially contribute to inspiratory muscle weakness.

The underlying cause of diaphragm muscle fiber weakness in PH is not clear. Both systemic as well as local factors may affect inspiratory muscle function in PH-patients (30). For instance, oxygen supply to the muscles may be reduced due to cardiac dysfunction and a reduction in cardiac output during exercise (17, 38, 40). However, no correlation between cardiac output and diaphragm muscle function was observed, suggesting that other factors may play a role.

Pro-inflammatory cytokines are elevated in the systemic circulation of PH-patients, which may affect the muscles (8, 13, 37). This may explain why peripheral muscle dysfunction is also observed in PH-patients (3, 4, 25, 31). Muscles are also sensitive to changes in activity and load, and remodel accordingly. PH-patients hyperventilate, or become hypercapnic, during exercise, at rest, and sometimes even during sleep, placing an increased demand on the inspiratory muscles (22, 28, 29, 33). The underlying cause of this hyperventilation is not completely clear, but it might be a reflection of increased sympathetic overdrive. The low end-tidal CO₂ (PetCO₂) as well as CO₂ arterial pressure in PH-patients (7, 16, 41) and also in our cohort of CTEPH-patients (table 1), suggest that PH-patients indeed ventilate more than needed.

We propose that a combination of both systemic as well as local factors may lead to diaphragm muscle weakness (30). Identification of these factors might also shed light on the finding that slow-twitch muscle fibers (lower maximal tension) are differently affected than fast-twitch muscle fibers (lower calcium sensitivity of force) in CTEPH-patients. The lower maximal tension in slow-twitch fibers in CTEPH-patients was associated with a reduction in the number of attached cross-bridges. This suggests that these fibers may have a lower concentration of contractile proteins. Indeed, analysis of the concentration of myosin in slow-twitch fibers revealed that this was significantly decreased in CTEPH diaphragm fibers (Fig.4). The magnitude of the reduction suggests that loss of contractile proteins is a major
mechanism underlying the development of contractile weakness in slow-twitch diaphragm fibers of CTEPH-patients.

**Clinical relevance**

In the current study we tested the ability of the fast skeletal troponin activator, CK-2066260, to restore submaximal diaphragm fiber strength. Upon exposure to CK-2066260, the contractile strength of fast-twitch diaphragm fibers of CTEPH-patients as well as of control subjects markedly improved at submaximal calcium concentrations (Fig. 5D and 5E). Since ~50% of fibers in the human diaphragm consists of fast-twitch fibers (18), fast skeletal troponin activators might significantly improve *in-vivo* diaphragm strength. Although during normal breathing slow-twitch diaphragm fibers are predominantly recruited, augmenting the contractility of fast-twitch diaphragm fibers might be beneficial during episodes of increased diaphragm activity, such as hyperventilation or dyspnea. In addition, fast troponin activators do not affect cardiac function (36), which would be an undesired side effect in PH, thereby strengthening the potential of these drugs. The analogue of CK-2066260, *tirasemtiv*, is currently under study in patients with amyotrophic lateral sclerosis.

The correlation between diaphragm muscle fiber maximal force and MIP in CTEPH-patients (Fig. 6) suggests that contractile dysfunction of diaphragm fibers contributes to inspiratory muscle dysfunction. The disparity between the reduction in diaphragm muscle fiber maximal tension (~15%) and *in vivo* inspiratory muscle function (~25%) suggests that extra-sarcomeric changes, neuromuscular transmission, neural input, and/or weakness of other inspiratory muscles might also contribute to *in vivo* inspiratory muscle weakness. However, when the findings from slow-twitch and fast-twitch muscle fibers are combined, reflecting total diaphragm function, a reduction of ~25% in submaximal tension is observed (Fig. 5C). A reduction of 25% in tension, at activation levels close to those *in vivo*, may very well be of clinical significance, in particular during activities that involve strenuous exercise.
We propose that weakening of individual diaphragm fibers is not unique to CTEPH-patients, but represents a pathophysiological process that is present in all forms of PH. Comparable values for MIP, as we observed in CTEPH-patients, were reported in patient cohorts consisting of idiopathic PH (32). Furthermore, pilot data from de Man et al. on end-stage PH-patients also showed a significant reduction in force generating capacity of diaphragm fibers (28). Thus, also in other forms of PH, weakening of diaphragm muscle fibers likely contributes to weakness of the inspiratory muscles.

Study limitations

For in vivo measures of inspiratory muscle function a MIP maneuver was used. This was a voluntary test and differences in patients’ effort can influence the results. However, it was previously shown that MIP is significantly lower in PH-patients, either measured by voluntarily maneuvers or by stimulation of the phrenic nerve (22). Furthermore, MIP was not determined in control subjects, and therefore caution is warranted when interpreting the MIP data from the CTEPH-patients. The values for MIP in the present group of CTEPH-patients were comparable to those determined in previous studies, studies in which MIP values in control subjects were also determined and were significantly higher than those in CTEPH-patients (22, 32). This suggests that in the present study the MIP maneuvers were executed properly, and that the lower values than predicted in the CTEPH-patients indeed reflect inspiratory muscle weakness.
REFERENCES


30. Manders E, Rain S, Bogaard H-J, Handoko ML, Stienen GJM, Vonk-Noordegraaf A,


FIGURE CAPTIONS

Figure 1: Stretch experiment - The slope of the instantaneous tension response to stretch ($\Delta T$) during maximal activation divided by length change ($\Delta L$) provides a measure of muscle fiber active stiffness, which is an estimate of the number of attached cross-bridges during activation. (□) represent a control muscle fiber; (•) represent a CTEPH muscle fiber. Note the steeper slope in the control fiber, indicating a higher number of attached cross-bridges. Measurements were included when a minimum of three fibers per fiber type per patient was reached.

Figure 2: No atrophy in the diaphragm muscle of CTEPH-patients - A. Examples of diaphragm muscle sections of a control and a CTEPH-patient stained for fast-twitch myosin heavy chain (blue), slow-twitch (black), and the plasma membrane (red). B. No significant differences in diaphragm cross sectional area (CSA), in slow-twitch and fast-twitch muscle fibers of CTEPH-patients (•) and controls (□) are observed. Data are presented as mean ± SEM. N indicates number of subjects studied.

Figure 3: Depressed contractile function of slow-twitch muscle fibers - A. Maximal tension is significantly lower in slow-twitch diaphragm muscle fibers of CTEPH-patients (•) than in controls (□). No difference is observed in fast-twitch muscle fibers. B. Diaphragm muscle fiber active stiffness is significantly lower in slow-twitch muscle fibers of CTEPH-patients than in controls. No change is observed in fast-twitch muscle fibers. C. The tension/stiffness ratio, a reflection of the force generated per cross-bridge, is not significantly different between groups. Data are presented as mean ± SEM, * p<0.05 vs. controls. N indicates number of subjects studied; numbers above indicate number of fibers measured.
Figure 4: Reduced MHC-concentration in slow-twitch muscle fibers. - A. Example of an acrylamide gel with myosin standards, single slow-twitch diaphragm fibers and a diaphragm homogenate. By comparing the intensity of the single muscle fiber bands to that of the MHC standard curve, we determined the amount of MHC present in the slow-twitch muscle fibers. B. MHC concentration was significantly lower in slow-twitch muscle fibers of CTEPH-patients compared to control subjects. Data are presented as mean ± SEM, * p<0.05 vs. controls. N indicates number of subjects studied.

Figure 5: Decreased calcium-sensitivity of force in fast-twitch muscle fibers. – A. Normalized force-[Ca^{2+}] relation of CTEPH-patients (▪) and controls (□) of slow-twitch and B. fast-twitch muscle fibers. A significant right-ward shift of the normalized force-[Ca^{2+}] relation is observed in fast-twitch muscle fibers of CTEPH-patients. C. Tension at [Ca^{2+}] of 0.63 μM is significantly lower in fast-twitch muscle fiber of CTEPH-patients. D. Normalized force-[Ca^{2+}] curves of fast-twitch muscle fibers with vehicle (1% DMSO) and after administration of 5μM CK-2066260. In both controls and CTEPH-patients CK-2066260 induces a significant left-ward shift of the normalized force-[Ca^{2+}] curves. E. The fast skeletal troponin activator CK-2066260 significantly improves submaximal tension generation at [Ca^{2+}] of 0.63μM in CTEPH-patients in fast-twitch muscle fibers; the tension of treated fibers of CTEPH-patients exceeds the tension of untreated control fibers. In slow-twitch muscle fibers no effect of CK-2066260 was observed. Data are presented as mean ± SEM, * p<0.05 vs. controls. N indicates number of subjects studied.

Figure 6: Correlation of maximal inspiratory pressure and diaphragm muscle force - Maximal inspiratory pressure (MIP) of CTEPH-patients correlates significantly with diaphragm muscle fiber maximal force.

Figure 7: Diaphragm and non-diaphragm muscle comparison in CTEPH patients - A. No significant differences in cross sectional area (CSA) of slow-twitch and fast-twitch fibers are
observed in the non-diaphragm muscle compared to the diaphragm muscle of CTEPH-patients.

B. Maximal tension is significantly lower in slow-twitch diaphragm muscle fibers than in non-diaphragm muscle of CTEPH patients. No difference is observed in fast-twitch muscle fibers. Data are presented as mean ± SEM, * p<0.05 vs. Non-DIA. N indicates number of subjects studied.
Table 1.

<table>
<thead>
<tr>
<th>Patients’ characteristics</th>
<th>CTRL (N=15)</th>
<th>CTEPH (N=13)</th>
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</thead>
<tbody>
<tr>
<td>Gender (M/F)</td>
<td>9 / 6</td>
<td>7 / 6</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>59 ± 12</td>
<td>56 ± 15</td>
</tr>
<tr>
<td>BMI</td>
<td>25 ± 3</td>
<td>26 ± 4</td>
</tr>
<tr>
<td>PaCO₂ (kPa)</td>
<td></td>
<td>4.5 ± 0.5</td>
</tr>
<tr>
<td>PetCO₂ (kPa)</td>
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<td>3.0 ± 0.3</td>
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<tr>
<td>FEV₁ (%)</td>
<td>88 ± 15</td>
<td>87 ± 15</td>
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<tr>
<td>VC (L)</td>
<td>4.0 ± 1.0</td>
<td>3.8 ± 0.7</td>
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<tr>
<td>FEV₁/VC (%)</td>
<td>70 ± 8</td>
<td>71 ± 10</td>
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<tr>
<td>mPAP (mmHg)</td>
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<td>48 ± 10</td>
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<tr>
<td>Cardiac output (L/min)</td>
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<tr>
<td>6MWT (m)</td>
<td>405 ± 128</td>
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</tr>
<tr>
<td>MIP (kPa) [% of pred.]</td>
<td>6.2 ± 2.4 [76%]</td>
<td></td>
</tr>
<tr>
<td>MEP (kPa) [% of pred.]</td>
<td>9.5 ± 3.1 [87%]</td>
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<tr>
<td>Tumor classification (N)</td>
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<tr>
<td>Other</td>
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</tr>
</tbody>
</table>

Values are mean ± SD. CTRL = Control; CTEPH = chronic thromboembolic pulmonary hypertension; BMI = body mass index; PaCO₂ = CO₂ arterial pressure (obtained at start surgery); PetCO₂ = end-tidal CO₂ tension; FEV₁ = forced expiratory volume in 1 second; VC = Vital Capacity, FEV₁/VC = Tiffeneau index, mPAP = mean pulmonary artery pressure; 6MWT = 6 minute walking test; MIP = maximal inspiratory pressure; MEP = maximal expiratory pressure. Other consisted of benign inflammation with necrosis, cysts, adenocarcinoma and chondrosarcoma.
### Table 2: Fiber type distribution

<table>
<thead>
<tr>
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<th>CTRL (N=15, n=142)</th>
<th>CTEPH (N=13, n=138)</th>
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</thead>
<tbody>
<tr>
<td>Slow-twitch</td>
<td>62</td>
<td>73</td>
</tr>
<tr>
<td>Type 2A</td>
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<td>61</td>
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<tr>
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<tr>
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<td>3</td>
</tr>
<tr>
<td>Type 2A/slow-twitch</td>
<td>6</td>
<td>1</td>
</tr>
</tbody>
</table>
Figure 1

Graph showing the relationship between changes in tension and changes in length. The main graph displays two sets of data points connected by lines, indicating a linear relationship. The inset graph provides a detailed view of the tension changes over time.
Figure 2

A  CONTROL DIAPHRAGM

B  CTEPH DIAPHRAGM

Cross Sectional Area [\(\mu m^2\)]

- SLOW-TWITCH: N=14, N=11
- FAST-TWITCH: N=13, N=11
Figure 5

A. Slow-twitch fibers

B. Fast-twitch fibers

C. Tension [mN/mm²]

D. fast-twitch fibers with CK-2066260

E. Tension [mN/mm²]
Figure 7

A

Cross Sectional Area [μm²]

N=10  N=11  N=11  N=11

SLOW-TWITCH  FAST-TWITCH

Non-DIA  DIA

B

Maximal tension [mN/mm²]

N=9  N=9  N=9  N=9

SLOW-TWITCH  FAST-TWITCH

*