Reduced diaphragm muscle contractility in patients with chronic thromboembolic pulmonary hypertension

This chapter is based on:
CHAPTER 4

4.1 INTRODUCTION

Pulmonary hypertension (PH) is a progressive disease and despite improvements in disease-targeted therapies, PH-patients remain symptomatic and have a reduced survival [61]. Symptoms include limited exercise capacity and dyspnea [127], which are not only related to cardiac dysfunction, but also to dysfunction of peripheral [5, 6, 82] and inspiratory muscles [2, 22, 66, 85, 99].

The capacity of the inspiratory muscles to generate pressure is reduced in PH-patients [66, 99]. In addition, the load on the inspiratory muscles might be increased since PH-patients hyperventilate during exercise, at rest and sometimes even during sleep [102, 127]. The underlying cause of inspiratory muscle weakness might be contractile dysfunction of the diaphragm, the main inspiratory muscle. Key determinants of diaphragm muscle function are the size and the contractility of individual diaphragm muscle fibers. Diaphragm muscle fiber size is reduced in animal models of PH as well as in biopsies of end-stage PH-patients [2, 22]. Furthermore, the force generating capacity of individual diaphragm muscle fibers is reduced in animal models of PH [2, 22, 85]. Previously, we suggested that the reduced diaphragm muscle fiber contractility observed in rats with PH is also present in PH-patients. However, based on the low number of patients in that study (N=2), a definite conclusion could not be drawn [22].

Here, we hypothesized that structural and functional changes in diaphragm muscle fibers occur and contribute to the inspiratory muscle weakness in PH-patients. To test this hypothesis, we measured in vivo inspiratory muscle function and ex vivo diaphragm muscle fiber contractility within the same patient. To determine ex vivo muscle contractility, biopsies of the diaphragm muscle are indispensable. For this reason, we focused on patients with operable chronic thromboembolic PH (CTEPH): during pulmonary endarterectomy the diaphragm was readily accessible and biopsies could be obtained. 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 calcium sensitivity of force.

4.2 METHODS

Subjects and respiratory muscle function testing

Diaphragm muscle biopsies were obtained from patients with chronic thromboembolic pulmonary hypertension (CTEPH, N=13) during a pulmonary thromboendarterectomy and from patients during elective thoracotomy for resection of small pulmonary tumor (CTRL, N=15). In addition, muscle biopsies of either the pectoralis major or rectus abdominis were obtained from the CTEPH patients (Non-diaphragm). 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 relaxing/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 rule out cachexia), primary lung disease (including COPD), neuromuscular disease and chronic use of corticosteroids.

Spirometry and maximal inspiratory and expiratory pressures were assessed in the CTEPH patients (N=9) 1-2 days prior to surgery as described previously [38]. In brief, patients were sitting in an upright position and breathed through a flanged mouthpiece. Maximal inspiratory pressure (MIP) was determined during a deep inspiration from functional residual capacity against a shutter. 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 manoeuvres, average standard deviation between consecutive manoeuvres was 0.9kPa.

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

**Histology**

To determine muscle 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 coverglass-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.

**Single muscle fiber contractile measurements**

Single muscle fibers (∼ 1.0mm 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 [85].

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 [85, 105, 106].
The composition of the relaxing solution (with a total ionic strength of 180 mM) consisted of 5.89 mM Na$_2$ATP, 6.48 mM MgCl$_2$, 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. The relaxing solution was set to a [Ca$^{2+}$] of 1 nM, whereas activating solutions ranging from 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 a EGTA concentration of 0.1 mM [85, 106].

Single fiber contractile experiments were performed as described previously [85]. 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 pre-activated by placing it in activating solution ([Ca$^{2+}$] 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 ellipsoid. 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-[Ca$^{2+}$] relationship, the fiber was placed for 1 minute in jump solution followed by activating solutions with incremental Ca$^{2+}$ concentrations ranging from 0.1 till 32 µM, and the isometric force generation was recorded. Force values at submaximal [Ca$^{2+}$] were normalized to the maximal force obtained at 32 µM [Ca$^{2+}$] to determine Ca$^{2+}$-sensitivity of the fiber expressed as EC$_{50}$, i.e. the [Ca$^{2+}$] at which 50% of maximal force is reached. The EC$_{50}$ was determined by fitting a modified Hill equation through the data points.

The effect of the fast-troponin activator CK-2066260 was tested in a subset of CTEPH-patients (N=3) and controls (N=3). A concentration of 5 µM of CK-2066260 was used based on previous studies with the same compound and similar tissue [58]. 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 (k$_{tr}$) 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 k$_{tr}$ was determined by fitting a double exponential through the force redevelopment curve.

Following the k$_{tr}$ 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. 4.1). 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.

Measurements were included when a minimum of three fibers per fiber type per patient was reached.
Figure 4.1: Stretch experiment - The slope of the instantaneous tension response to stretch (△T) during maximal activation divided by length change (△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.

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. A small strip of the aluminium clip was left on the fibers on each side, and the fiber was placed in 25 μL of SDS sample buffer. MHC isoform composition and content was determined by SDS-PAGE as described previously [85]. 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 25 μL SDS buffer) based on the standard curve. MHC concentration was calculated by dividing total MHC content by 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. Fibres that co-expressed both slow-twitch and fast-twitch MHC isoforms were excluded from further analysis (cut off value of 75% of one type).
Table 4.1: Patients’ characteristics

<table>
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<th>CTRL (N=15)</th>
<th>CTEPH (N=13)</th>
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<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>
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<tr>
<td>BMI</td>
<td>25 ± 3</td>
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<tr>
<td>FEV₁ (%)</td>
<td>88 ± 16</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>
</tr>
<tr>
<td>FEV₁/VC (%)</td>
<td>70 ± 8</td>
<td>71 ± 10</td>
</tr>
<tr>
<td>mPAP (mmHg)</td>
<td></td>
<td>48 ± 10</td>
</tr>
<tr>
<td>Cardiac output (L/min)</td>
<td>4.3 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>6MWT (m)</td>
<td>405 ± 128</td>
<td></td>
</tr>
<tr>
<td>MIP (kPa)</td>
<td>6.2 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>MEP (kPa)</td>
<td>9.5 ± 3.1</td>
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Values are mean ± SD. CTRL = Control; CTEPH = chronic thromboembolic pulmonary hypertension; BMI = body mass index; 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.

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 [18, 20, 48, 85]. If data could not be analysed with multilevel analysis an independent student-t test or Mann Whitney U test was used on the averages per patient. 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.

4.3 RESULTS

Subjects Characteristics

Patients’ characteristics, pulmonary function and respiratory muscle strength are shown in table 4.1. No differences between CTEPH and control patients were observed with regard to gender, age, body mass index, and pulmonary function.
Figure 4.2: 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.

Histology

The CSA of fast-twitch and slow-twitch diaphragm fibers was assessed in control subjects (N=15) and CTEPH-patients (N=11). Typical examples are shown in figure 4.2A. No significant difference in CSA was observed between groups in both slow-twitch and fast-twitch muscle fibers (Fig. 4.2B). 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 271 individual fibers of CTEPH-patients (N=13) and controls (N=15) were manually isolated from the diaphragm biopsies and used for contractile measurements. Due to technical difficulties, not all parameters could be measured in all fibers. The number of patients per parameter is indicated in the figures.

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. A typical force trace is shown in figure 4.3A. Maximal tension of slow-twitch muscle fibers was significantly lower in CTEPH-patients than in controls (Fig. 4.3B). No difference in maximal tension was observed in fast-twitch muscle fibers.

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

First, to estimate $\alpha_{fs}$ we measured the rate of force redevelopment ($k_{tr}$) during maximal activation. No significant difference in $k_{tr}$ was observed between groups in both slow-twitch (CTRL vs. CTEPH: 5.1 ± 0.1 vs. 5.2 ± 0.2 [s$^{-1}$]) and fast-twitch (CTRL vs. CTEPH: 14.2 ± 0.6 vs. 14.1 ± 0.8 [s$^{-1}$]) muscle fibers, suggesting that the $\alpha_{fs}$ was unaltered.

Second, to estimate the number of available cross-bridges, we measured MHC concentration in the single muscle fibers (Fig. 4.4A and B). In CTEPH-patients a significant reduction in MHC-concentration was observed in both slow-twitch and fast-twitch muscle fibers (Fig. 4.4C), indicating a reduction in the number of available cross-bridges.

The functional consequence of these changes was determined by measuring the active stiffness of the fibers during activation. By imposing small length changes on the fiber during maximal activation (see methods Fig. 4.1) the number of attached cross-bridges during activation can be estimated. This number is determined by both $\alpha_{fs}$ and the number of available cross-bridges. A reduction in active stiffness was observed in slow-twitch muscle fibers of CTEPH-patients (Fig. 4.5A) while no change is observed in fast-twitch muscle fibers. This suggests that fast-twitch, but not slow-twitch, muscle
Figure 4.4: Reduction in MHC concentration - A. Example of an acrylamide gel with myosin standards, a diaphragm homogenate, and single muscle fibers of control and CTEPH-patients expressing various myosin heavy chain (MHC) isoforms. B. By comparing the intensity of the single muscle fiber bands to that of the MHC standard bands, we determined the amount of MHC present in the single muscle fibers. C. MHC concentration is significantly lower in both slow-twitch and fast-twitch muscle fibers of CTEPH-patients (■) than in controls (□). Data are presented as mean ± SEM, ∗ p<0.05 vs. control. N indicates number of subjects studied.

Figure 4.5: Reduction in the number of available cross-bridges - A. 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. B. 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. control. N indicates number of subjects studied.
fibers are able to compensate for the reduction in MHC.

Finally, we estimated the force generated per cross-bridge by calculating the tension/stiffness ratio. No significant difference was observed between groups (Fig. 4.5B), indicating that the reduction in maximal tension was proportional to the reduction in active stiffness.

**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 \([\text{Ca}^{2+}]\) and determined the \(\text{Ca}^{2+}\)-sensitivity of force. As shown in figure 4.6A, no shift in the force-[\(\text{Ca}^{2+}\)] curve was observed in slow-twitch muscle fibers of CTEPH-patients, indicating unaltered \(\text{Ca}^{2+}\)-sensitivity of force. In fast-twitch muscle fibers of CTEPH-patients a right-ward shift of the force-[\(\text{Ca}^{2+}\)] relation was observed (Fig. 4.6B), indicating reduced \(\text{Ca}^{2+}\)-sensitivity of force. The \([\text{Ca}^{2+}]\) 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 [\(\mu\text{M}\)], 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 [\(\mu\text{M}\)]). As a result, the tension (i.e. force per CSA) of fast-twitch muscle fibers was significantly reduced at \([\text{Ca}^{2+}]\) of 0.63 \(\mu\text{M}\); a concentration close to the EC\(_{50}\) (Fig. 4.6C).

Next, we tested the ability of the fast skeletal troponin activator CK-2066260 to improve the contractility at submaximal \([\text{Ca}^{2+}]\) in fast-twitch muscle fibers in a subset of CTEPH-patients (N=3) and control subjects (N=3). Previous work from our group showed that 5 \(\mu\text{M}\) of CK-2066260 yields a near-maximal effect [58]; therefore, this concentration was used in the present study. As CK-2066260 specifically targets fast troponin C [118], no effect on the \(\text{Ca}^{2+}\)-sensitivity of force in slow-twitch muscle was observed (Fig. 4.7A). However, in fast-twitch muscle fibers - the fiber type that showed a reduced \(\text{Ca}^{2+}\)-sensitivity of force in CTEPH-patients - 5 \(\mu\text{M}\) CK-2066260 significantly increases the \(\text{Ca}^{2+}\)-sensitivity of force in both control and CTEPH-patients (EC\(_{50}\) CTRL: DMSO vs. CK 0.70 ± 0.03 vs. 0.10 ± 0.01 [\(\mu\text{M}\)], p<0.05. EC\(_{50}\) CTEPH: DMSO vs. CK 0.89 ± 0.06 vs. 0.16 ± 0.01 [\(\mu\text{M}\)], p<0.05) (Fig. 4.7B). As a result, tension at \([\text{Ca}^{2+}]\) of 0.63 \(\mu\text{M}\) is significantly increased in fast-twitch muscle fibers of CTEPH-patients during exposure to CK-2066260 (Fig. 4.6D).

**In vivo inspiratory muscle function**

The average MIP measured in CTEPH-patients (6.2 ± 2.4 kPa, N=9) 1-2 days prior to pulmonary endarterectomy, was comparable to previously reported values in PH-patients (Table 4.1) [66, 99]. We sought to find correlations between MIP and diaphragm muscle fiber contractility and size (Table 4.2). For these correlations we pooled both fiber types, as MIP is a reflection of the contractile strength of all diaphragm fibers. Maximal diaphragm muscle fiber force showed a strong correlation with MIP (Fig. 4.8A). Both diaphragm muscle fiber CSA and maximal tension (i.e. force normalized to CSA) contribute to maximal force, but neither significantly correlated with MIP (Table 4.2).
Figure 4.6: Decreased calcium-sensitivity of force in fast-twitch muscle fibers. - A. Normalized tension - [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. The fast troponin activator CK-2066260 (CK) significantly improves submaximal tension generation at [Ca^{2+}] of 0.63 µM) in CTEPH-patients; the tension of treated fibers of CTEPH-patients exceeds the tension of untreated (DMSO). Data are presented as mean ± SEM, * p<0.05 vs. donor. N indicates number of subjects studied.

This suggests that a combination of both affects MIP. The maximal force of diaphragm muscle fibers did not significantly correlate with 6 minute walking test (6MWT, N=12) (Fig. 4.8B), but a trend was observed (p=0.06). Finally, hemodynamic parameters (e.g. mPAP, CO and PVR) did not correlate with MIP or with the maximal force of diaphragm fibers (Table 4.2).

4.4 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.
Figure 4.7: Increased Ca\textsuperscript{2+}-sensitivity of force with CK-2066260 in fast-twitch muscle fibers - A. Normalized tension-[Ca\textsuperscript{2+}] curves of slow-twitch and B. fast-twitch muscle fibers with vehicle (1% DMSO) and after administration of 5 \(\mu\)M CK-2066260. In slow-twitch muscle fibers no effect of CK was observed. In fast-twitch muscle fibers both in controls and CTEPH-patients CK induced a significantly left-ward shift. Data are presented as mean ± SEM, * p<0.05 vs. DMSO. N indicates number of subjects studied.

Figure 4.8: Correlation of maximal inspiratory pressure and diaphragm muscle force - A. Maximal inspiratory pressure (MIP) of CTEPH-patients correlates significantly with diaphragm muscle fiber maximal force. B. Maximal force is not significantly correlated to 6 minute walking test (6MWT).

2. the calcium sensitivity of force is reduced in fast-twitch diaphragm muscle fibers of CTEPH-patients, which could be restored with 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.
Table 4.2: Correlations diaphragm function

<table>
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<th>Correlation</th>
<th>$R^2$</th>
<th>p-value</th>
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<tr>
<td>MIP · Maximal tension</td>
<td>0.21</td>
<td>0.22</td>
</tr>
<tr>
<td>MIP · CSA</td>
<td>0.41</td>
<td>0.09</td>
</tr>
<tr>
<td>MIP · 6WMT</td>
<td>0.24</td>
<td>0.18</td>
</tr>
<tr>
<td>MIP · mPAP</td>
<td>0.25</td>
<td>0.17</td>
</tr>
<tr>
<td>MIP · CO</td>
<td>0.03</td>
<td>0.67</td>
</tr>
<tr>
<td>MIP · PVR</td>
<td>0.008</td>
<td>0.81</td>
</tr>
<tr>
<td>Maximal force · mPAP</td>
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<td>0.44</td>
</tr>
<tr>
<td>Maximal force · CO</td>
<td>0.001</td>
<td>0.92</td>
</tr>
<tr>
<td>Maximal force · PVR</td>
<td>0.04</td>
<td>0.50</td>
</tr>
</tbody>
</table>

MIP = maximal inspiratory pressure [kPa]; CSA = cross sectional area [mm$^2$]; 6MWT = 6 minute walking test [m]; mPAP = mean pulmonary artery pressure [mmHg]; CO = cardiac output [L/min]; PVR = pulmonary vascular resistance [dynes · sec · cm$^{-5}$].

Diaphragm muscle weakness in PH

The imbalance between the demand placed on the inspiratory muscles, and the capacity of the inspiratory muscles to generate pressure, could result in the sensation of dyspnea [87]. Previous work revealed that the demand placed on the inspiratory muscles is increased in PH-patients [102, 127]. In addition, the pressure generating capacity of the inspiratory muscles is decreased in PH-patients, which might be even more impaired in CTEPH-patients [66, 99]. Importantly, the average MIP (6.2kPa) measured in the present study was comparable to that previously reported (~6kPa), and is lower than the MIP of control subjects (~8kPa) [66, 99]. This indicates that our cohort of CTEPH-patients suffered from inspiratory muscle weakness. The pathophysiology that underlies this weakness was unknown. The uniqueness of the present study lies in the combined measurements of in vivo and ex vivo inspiratory muscle contractility in individual CTEPH-patients.

The diaphragm is the main muscle of inspiration, and its activation involves a complex sequence of physiological phenomena, including phrenic nerve stimulation, neuromuscular transmission, and diaphragm muscle fiber contraction. Assessment of MIP cannot discriminate between the relative contributions of these phenomena. Based on data from animal models of PH [2, 22, 85], we hypothesized that structural and functional changes in the diaphragm muscle fibers might play a role.

For the evaluation of diaphragm muscle fibers of PH-patients, biopsies are indispensable. These biopsies are small pieces of muscle obtained from the middle of the diaphragm. Hence, the fiber ends are not sealed by tendons, which disrupts normal excitation-contraction coupling. Therefore, we permeabilized the muscle fibers and measured their contractile properties by exposing the fibers to exogenous calcium and measuring the force response. These experiments revealed that the contractile strength
of diaphragm muscle fibers of CTEPH-patients is significantly reduced. The reduction is specific for slow-twitch muscle fibers, which occupy 55% of the total muscle fiber population in the human diaphragm, and which are typically recruited during normal breathing [89]. This weakness is, at least partly, caused by a reduction in the concentration of the major contractile protein myosin (Fig 4.4). A significant decrease in myosin was also observed in fast-twitch muscle fibers. How these fibers compensate for this reduction in myosin is unclear, but might involve changes in the rate of cross-bridge detachment [121].

The correlation between diaphragm muscle fiber maximal force and MIP in CTEPH-patients (Fig. 4.8A) suggests that diaphragm contractile dysfunction contributes to inspiratory muscle dysfunction, and maybe also to dyspnea. 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.

Besides a decrease in maximal force generating capacity, we also found a reduction in the Ca\(^{2+}\)-sensitivity of force in fast-twitch muscle fibers of the diaphragm in CTEPH-patients. This is an important finding, as the diaphragm in vivo is typically activated at submaximal firing rates, which generate submaximal cytosolic [Ca\(^{2+}\)]. The rightward shift of the normalized force-[Ca\(^{2+}\)] curve indicates that at a specific [Ca\(^{2+}\)], less tension is generated in diaphragm fibers of CTEPH-patients. A significant reduction in tension of ~25% was observed at submaximal [Ca\(^{2+}\)] when both slow- and fast-twitch fibers were combined (data not shown).

We observed no reduction in the CSA of diaphragm muscle fibers of CTEPH-patients, in contrast to significant reduction in CSA reported previously in PH-patients [22]. This discrepancy may be explained by the fact that the end-stage PH-patients in that study were much more severely hemodynamically compromised and most likely suffered from more severe inspiratory muscle weakness [31]. The CTEPH-patients studied here were selected for pulmonary endarterectomy, an advanced surgical intervention that is performed only on stable patients. In non-failing PH-rats no reduction in diaphragm muscle fiber CSA was observed [22]. Therefore, we propose that reductions in diaphragm fiber size only occur in end-stage disease. Interestingly, a reduction in myosin concentration in diaphragm muscle fibers of CTEPH-patients was observed (Fig.4.4), suggesting that atrophic processes were activated. Thus, the reduction in myosin concentration likely constitutes an initial step towards diaphragm muscle fiber atrophy in CTEPH-patients.

The underlying cause of diaphragm muscle fiber weakness in PH is not completely clear. It has been suggested that the increased ventilatory drive observed in PH-patients places an increased burden on the diaphragm muscle [22, 66, 85, 102]. Previous studies have shown contractile dysfunction in chronically over-stimulated skeletal muscles [72]. In addition, similar changes in the diaphragm muscle were observed in chronic obstructive pulmonary disease and chronic heart failure [60, 98, 105, 142], conditions that are
also associated with increased inspiratory muscles activity. However, it should be noted
that peripheral muscle dysfunction has also been observed in PH-patients, which could
be an indication of generalized systemic muscle weakness [5, 6, 82].

**Potential therapeutic opportunities**

An approach to improve inspiratory muscle function could be exercise training (ExT) and
inspiratory muscle training (IMT). Recently, improvements in twitch mouth pressure and
6MWT have been found after 15 weeks of a combined IMT and ExT program [65, 97].
Similar improvements have been found in chronic heart failure (CHF) patients in a
randomized trial of IMT alone [16]. Furthermore, there are indications in CHF-patients
that IMT and ExT also reduces sympathetic drive [96]. Most importantly, CHF and
PH-patients report a better quality of life and decreased sensation of dyspnea on exertion
after IMT [16, 65, 97].

Based on these recent findings, improving inspiratory muscle function pharmaco-
logically could be another therapeutic approach. In the present study, we tested the
ability of a fast skeletal troponin activator CK-2066260 to restore submaximal force in
fast-twitch muscle fibers. CK-2066260 slows the dissociation rate of calcium from tro-
ponin C, leading to a longer open conformation of the troponin/tropomyosin complex
to enhance cross-bridge formation at a given calcium concentration [118]. Submaximal
tension generation of CK-2066260 treated diaphragm fibers of CTEPH-patients exceeds
the levels observed in untreated fibers of control subjects (Fig. 4.6D). This illustrates the
therapeutic potential of fast skeletal troponin activators. Importantly, CK-2066260 is
selective for fast-skeletal troponin C, and therefore has no effect on slow-skeletal muscles
(Fig.4.7A) or cardiac muscle [118]. Consequently, undesirable side-effects on the heart
are unlikely to occur. CK-2066260 is an analogue of tirasemtiv (formerly CK-2017357),
which is in clinical trials (e.g. NCT01709149) for amyotrophic lateral sclerosis patients.

**Study limitations**

For in vivo measures of inspiratory muscle function a voluntary 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 [66]. In
addition, similar values of MIP were obtained in this cohort of patients compared with
previous studies [66, 99], suggesting that MIP maneuvers were executed properly.

Diaphragm muscle biopsies were obtained during pulmonary endarterectomy, during
which the patient is placed on cardiopulmonary bypass and cooled to a core temperature
of ~18°C [128]. As this procedure alone might affect muscle function, we also obtained a
biopsy of the pectoralis major muscle or rectus abdominus muscle in the same patient,
~5 minutes after obtaining the diaphragm muscle biopsy. An extensive comparison
between the diaphragm muscle and non-diaphragm muscle is shown in figures 4.9 - 4.11.
Figure 4.9: A. No significant differences in cross sectional area (CSA), in slow-twitch and fast-twitch muscle fibers of the non-diaphragm muscle compared to diaphragm muscle of CTEPH-patients were observed. B. Maximal tension was significantly lower in slow-twitch diaphragm muscle than in non-diaphragm muscle of CTEPH patients. No difference was observed in fast-twitch muscle fibers. Data are presented as mean ± SEM, * p<0.05 vs. donor. N indicates number of subjects studied.

Maximal tension of non-diaphragm muscle fibers was significantly higher compared to diaphragm muscle fibers of CTEPH-patients. 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 diaphragm muscle contractile function obtained during pulmonary endarterectomy.

Conclusion

The present study indicates that diaphragm muscle fiber weakness contributes to reduced inspiratory muscle strength in CTEPH-patients. The fast skeletal troponin activator CK-2066260 is able to markedly augment force generation in diaphragm fibers of CTEPH-patients. These findings provide a rationale to test diaphragm-centered therapeutic strategies, aimed at improving diaphragm contractility and quality of life in PH-patients.
Figure 4.10: A. Calcium sensitivity, expressed as EC$_{50}$, was not significantly different in both fiber types. However, a strong trend of lower EC$_{50}$ was observed in fast-twitch muscle fibers of the diaphragm. B. The rate of force redevelopment ($k_{tr}$) was significantly lower in slow-twitch muscle fiber of the diaphragm compared to non-diaphragm muscle. No difference was observed in fast-twitch muscle fibers between groups. Data are presented as mean ± SEM, * p<0.05 vs. donor. N indicates number of subjects studied.

Figure 4.11: A. Diaphragm muscle fiber active stiffness was significantly lower in slow-twitch muscle fibers compared to non-diaphragm muscle. No change was observed in fast-twitch muscle fibers. B. The tension/stiffness ratio was significantly lower in both slow-twitch and fast-twitch muscle fibers of the diaphragm compared to non-diaphragm muscle. C. Myosin heavy chain (MHC) concentration was not significantly different between groups. D. Maximal force per half sarcomere MHC content was not significantly different in both fibers types. Data are presented as mean ± SEM, * p<0.05 vs. donor. N indicates number of subjects studied.