Diaphragm weakness in pulmonary arterial hypertension: role of sarcomeric dysfunction

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Submitted 25 April 2012; accepted in final form 6 September 2012

Diaphragm weakness in pulmonary arterial hypertension: role of sarcomeric dysfunction. Am J Physiol Lung Cell Mol Physiol 303: L1070–L1078, 2012. First published September 7, 2012; doi:10.1152/ajplung.00135.2012.—We previously demonstrated that diaphragm muscle weakness is present in experimental pulmonary arterial hypertension (PH). However, the nature of this diaphragm weakness is still unknown. Therefore, the aim of this study was to investigate whether changes at the sarcomeric level contribute to diaphragm weakness in PH. For this purpose, in control rats and rats with monocrotaline-induced PH, contractile performance and myosin heavy chain content of demembranated single diaphragm fibers were determined. We observed a reduced maximal tension of 20% (P < 0.05), whereas tension cost was preserved in type 2X and 2B diaphragm fibers in PH compared with control. The reduced maximal tension was associated with a reduction of force generated per half-sarcomeric myosin heavy chain content. Additionally, reduced Ca2+ sensitivity of force generation was found in type 2X fibers compared with control, which could exacerbate diaphragm muscle weakness at submaximal activation. No changes in maximal tension and Ca2+ sensitivity of force generation were observed in fibers from the nonrespiratory extensor digitorum longus muscle. Together, these findings indicate that diaphragm weakness in PH is at least partly caused by sarcomeric dysfunction, which appears to be specific for the diaphragm.

single fiber, cross bridge cycling kinetics; Ca2+ sensitivity; myosin heavy chain

PULMONARY ARTERIAL HYPERTENSION (PH) is characterized by a progressive increase in pulmonary vascular resistance, ultimately leading to right heart failure and death. Patients with PH hyperventilate at rest, during exercise, and sometimes even during sleep (19, 27). As a consequence, inspiratory muscle activity increases substantially, which may ultimately lead to overloading of the inspiratory muscles and respiratory muscle weakness.

Recent studies have found markedly lower maximal inspiratory pressures in patients with PH compared with control subjects (19, 25). This indicates that the force-generating capacity of the inspiratory muscles is impaired. The presence of inspiratory muscle weakness in PH was further supported by recent work from our group on a rat model for PH, which revealed a significant reduction in twitch and maximal tetanic force generation of the diaphragm, the main muscle of inspiration (8). Data from De Man et al. (8) also suggests a significant reduction of maximal tension in diaphragm fibers of two patients with PH.

Thus evidence is accumulating that the diaphragm is weakened in PH. However, the nature of this diaphragm weakness is unknown and might involve changes at the level of sarcoplasmic reticulum calcium cycling or content or at levels downstream in the excitation-contraction coupling process, i.e., at the sarcomeric level. Recent studies indicate that sarcomeric function of the diaphragm is sensitive to changes in contractile activity. For instance, diaphragm unloading during mechanical ventilation and denervation is associated with rapid sarcomeric dysfunction (e.g., lower active and passive tension and decreased myosin content) and alterations in ATP consumption (10, 16, 33, 37). On the other hand, sarcomeric dysfunction in the diaphragm is also reported in conditions associated with increased activity, such as chronic obstructive pulmonary disease (23, 30) and heart failure (17).

Therefore, in the present study, we hypothesized that sarcomeric dysfunction contributes to diaphragm weakness in PH. To test this hypothesis, the contractile performance of demembranated (skinned) single diaphragm fibers of PH and control rats was determined. In skinned fibers, the membranous structures are made permeable while leaving the sarcomeres intact. By exposing these cells to exogenous calcium, sarcomeric function could be studied without confounding effects of sarcoplasmic reticulum functioning. In addition, myosin heavy chain (MHC) content in these fibers was measured to assess whether loss of this major contractile protein contributes to sarcomeric dysfunction. Our findings reveal that the force-generating capacity of diaphragm fibers from PH rats is reduced, resulting in a lower force per cross bridge, indicating that sarcomeric dysfunction contributes to diaphragm weakness in PH.

MATERIALS AND METHODS

Animals

The experiments were performed on tissue that was from the same rats (male Wistar rats, 150–175 g; Harlan, Horst, the Netherlands) as used in the study of De Man et al. (8). Briefly, PH was induced by a single subcutaneous injection of 60 mg/kg monocrotaline (Sigma-Aldrich, Zwijndrecht, the Netherlands) dissolved in sterile saline, and the control group was injected with saline only (control n = 7; PH n = 7). When right heart failure (RHF) developed, defined as >5% weight loss per day (and/or respiratory distress, cyanosis, or lethargy), rats were hemodynamically evaluated by echocardiography and invasive right ventricular pressure measurements (14). Subsequently, the diaphragm and extensor digitorum longus (EDL) muscle were excised. In a separate cohort of 10 rats (5 control; 5 PH), breathing frequencies...
were determined by means of telemetry (15). At each time point, 10 subsequent recordings of 10 s of ventricular pressure were analyzed. A fast Fourier transform was used to determine the dominant frequencies in the signal. From the single side amplitude spectrum, two dominant frequencies could be distinguished: one corresponding to heart rate and one to breathing frequency (for a typical example, see Fig. 1, A and B).

**Tissue Preparation**

Fresh muscle strips, dissected from the excised diaphragm and EDL muscles, were placed overnight at 5°C in relaxing solution (for composition, see below) containing 1% Triton X-100 to permeabilize the membranes. Subsequently, the specimens were washed overnight with relaxing solution, then placed in a 50% glycerol/relaxing solution (vol/vol), and stored at −20°C until further use.

**Single Muscle Fiber Contractile Measurements**

Single muscle fibers (~1.0 mm in length) were isolated from the diaphragm and EDL muscle strips using micro forceps. The fiber ends were attached to aluminum foil clips and mounted on a single-fiber apparatus (Aurora Scientific, Aurora, Ontario, Canada), which was placed on top of an inverted microscope. One end of the fiber was attached to a force transducer (model 403A, Aurora Scientific), whereas the other end was attached to a servomotor (315C, Aurora Scientific). Fibers that appeared damaged during microscopic examination were excluded from the study. The number of excluded fibers did not differ per group. All measurements were performed at 20°C (29, 30).

The composition of relaxing solution (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. The negative logarithm of the K-propionate, 100 mM BES, 6.97 mM EGTA, and 14.5 mM CrP with (RHF). Data are presented as means between 3–7 Hz indicates heart rate. The rate constant of force redevelopment (k_d) was measured by rapidly releasing the fiber by ~20% followed by a restretch to its original length after 30 ms. (4, 32) This release detaches all myosin heads attached to actin, and subsequently force redevelops. The k_d was determined by fitting a single exponential through the force redevelopment curve.

To determine the tension-pCa relationship, the fiber was placed in solutions with increasing Ca^{2+} concentrations (pCa 7.0, 6.2, 6.0, 5.8, 5.4, and 4.5), and the isometric force generation was recorded. The Ca^{2+} concentration required for half-maximal activation (pCa_{50}), as a measure of Ca^{2+} sensitivity, was determined by fitting a modified Hill equation through the data points.

**Determination of MHC Isoform Composition and MHC Content Per Half-Sarcomere**

At the end of the single fiber contractile protocol, the fibers were detached from the force transducer and servomotor and placed in 25 μL of SDS sample buffer containing 62.5 mM Tris-HCl, 2% (wt/vol) SDS, 10% (vol/vol) glycerol, and 0.001% (wt/vol) bromophenol blue at a pH of 6.9. MHC isoform composition and content by SDS-PAGE was determined, as described by Geiger et al. (11) with minor modifications (17). Briefly, samples were denatured by boiling for 2 min. The stacking gel contained 4% acrylamide (pH 6.8), and the separating gel contained 7% acrylamide (pH 8.8) with 30% (vol/vol) glycerol. A homogenate of control rat 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 rat diaphragm single fibers. Sample volumes of 20 μL were loaded per lane. The gels were silver stained according to the procedure described by Oakley et al. (28). Note that the identification of MHC isoforms in rat diaphragm was previously validated using MHC isoform antibodies (11) and that the technique described above results in complete extraction of MHC from the diaphragm fibers (11). For the identification of hybrid fibers, a cutoff value was used; a fiber was considered hybrid only if the dominant MHC isoform constituted <75% of total MHC content.

After we stained the gel, we scanned it with an image densitometer, and optical densities of the electrophoretic bands were quantified. Background staining was subtracted from the density of the electrophoretic bands to determine the brightness-area product for each diaphragm muscle fiber. The relationship between the brightness-area...
product and MHC content was linear across a range from 0.01 to 0.25 μg (16). The MHC content in the loaded 20-μl SDS buffer was determined from the standard curve. Subsequently, the total MHC content of the fiber (in 25 μl SDS buffer) was determined (33). MHC concentration was determined by dividing total MHC content by fiber volume. MHC content per half-sarcomere, at a sarcomere length of 2.5 μm, was calculated by dividing total MHC content of the fiber by the amount of half-sarcomeres [2 × length of fiber (μm)]/2.5 μm]. Maximal force generation at pCa 4.5 was divided by MHC content per half-sarcomere to determine the force per half-sarcomere MHC content.

ATPase Measurements

The rate of ATP consumption during maximal isometric tension was measured using a NADH-linked fluorescence technique enzymatic assay, as described by Stienen et al. (36) and by Sieck and Prakash (32), who employed this technique for the first time on diaphragm fibers. Briefly, Phospho(enol)-pyruvate (PEP), pyruvate kinase (PK), and lactate dehydrogenase (LDH) were added to activating, preactivating, and relaxing solutions to catalyze the following reactions:

\[
\begin{align*}
\text{ATP} & \rightarrow \text{ADP} + P_i \\
\text{ADP} + \text{PEP} & \rightarrow \text{pyruvate} + \text{ATP} \\
\text{pyruvate} + \text{NADH} & \rightarrow \text{lactate} + \text{NAD}^+ 
\end{align*}
\]

These equations illustrate that, when ATP is consumed, NADH is converted to NAD+. Because NADH is fluorescent, whereas NAD+ is not, the rate of decline in NADH absorbance is proportional to the rate of ATP consumption.

Permeabilized muscle fiber bundles (diameter ~100 μm) of the diaphragm were mounted between a force transducer and length motor in relaxing solution, and sarcomere length was set at 2.5 μm using the diffraction pattern of a He-Ne laser beam. The bundle was incubated in relaxing solution for 3 min, preactivating solution for about 3 min and then in activating solution (pCa 4.5) until a steady force level was attained. During activation, NADH absorbance was measured by a near UV-light beam, which passes underneath the fiber bundle while the solution was continuously mixed. The bundle is then placed back in relaxing solution, and the absorbance signal of NADH was calibrated by adding a known amount of ADP. Basal ATP consumption was measured in relaxing solution (pCa 9.0) and subtracted from ATP consumption during activation.

Oxidative Stress Measurements

To evaluate the degree of contractile protein oxidation in the diaphragm muscle of PH rats, nitrotyrosination and protein carbonylation were studied.

Nitrotyrosination. Peroxynitrite formation was evaluated in the diaphragm muscle by detection of nitrotyrosine residues by performing Western immunoblotting with a monoclonal anti-nitrotyrosine antibody of high specificity (clone 1A6; Upstate Biotechnology, Lake Placid, NY), as discussed previously (40). Crude muscle homogenate proteins (10 μg) were heated for 5 min at 95°C in sample buffer and then separated by electrophoresis (200 V for 1 h) on 10% SDS-PAGE. Proteins were transferred electrophoretically (100 V, 300 mA for 1 h) to methanol-presoaked polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were subsequently incubated with primary monoclonal antibodies against nitrotyrosine in 0.1% bovine serum albumin in PBS with 1% Tween 20 (PBST). After three 10-min washes with PBST on a shaker, the PVDF membranes were incubated with a secondary antibody (polyclonal anti-mouse IgG horseradish peroxidase conjugated) for 1 h at room temperature. The membranes were finally washed twice for 10 min with PBST.

Afterward, protein bands were visualized using an enhanced chemiluminescence detection kit (Amersham Biosciences Europe, Uppsala, Sweden). The blots were scanned with an imaging densitometer, and optical densities were quantified using GeneTools software (Syngene, Cambridge, UK).

Protein carbonylation. Protein extraction was carried out as described previously by Canton et al. (5), and aliquots of this extract were assayed for protein concentration (Bradford Bio-Rad Protein Assay; Bio-Rad Laboratories, Hercules, CA). The Oxyblot Oxidized Protein Detection Kit was purchased from Chemicon (Temecula, CA), and 2,4-dinitrophenyl hydrazine (DNPH) derivatization was carried out for 15 min following manufacturer’s instructions on 15 μg of protein. The same samples were also prepared in the absence of DNPH as a control. One-dimensional electrophoresis was carried out on a 15–4% gradient SDS-PAGE gels with 6 μg of derivatized-protein loaded per lane. Proteins were transferred to nitrocellulose membranes at 100 mA constant current for 90 min. The membrane was subsequently incubated for 2 h at room temperature with anti-DNP (1:100, Chemicon). The blot was washed three times and was subsequently incubated for 1 h with Goat Anti-Rabbit IgG (horseradish peroxidase conjugated, 1:300, Chemicon). The blot was developed using a chemiluminescence detection kit. The blot was scanned with an imaging densitometer, and optical densities were quantified.

Statistical Methods

Maximal tension, MHC concentration, pCa50, and tension cost in single fibers were tested with a multilevel analysis to correct for nonindependence of successive measurements per animal (MLwiN, 2.02.3; Centre for Multilevel Modeling, Bristol, UK) (6, 7, 14). Nitrotyrosine and oxyblot optical density were analyzed by independent t-test. Two-way repeated-measure ANOVA was used to analyze the differences in breathing frequency and tension-pCa curves between control and PH, with Bonferroni post hoc tests. A P value of <0.05 was considered significant.

RESULTS

Animal Characteristics

Animal characteristics are as reported previously by De Man et al. (8). All PH rats had clear signs of right ventricular (RV) dysfunction (decreased cardiac output, tricuspid annular plane excursion), pulmonary vascular remodeling (increased RV systolic pressure and pulmonary vascular resistance), and increased RV remodeling (increased RV wall thickness, RV end diastolic diameter) compared with controls. As can be seen in Fig. 1C, breathing frequencies in PH rats were significantly increased starting 5 days before the development of RHF.

Diaphragm Single Muscle Fiber Contractility

In total 103 single fibers from the diaphragm muscle of control and PH rats were used for contractile measurements. These fibers were grouped per fiber type: I, 2A, 2X, and 2B. Note that, due to the low number of 2A fibers (n = 4 in control, n = 3 in PH), results from this fiber type are omitted from the figures. Fibers that coexpressed different MHC isoforms (n = 7 for PH and n = 4 for controls) were excluded from further analysis. In some fibers, not all parameters could be measured due to technical circumstances. Therefore, the number of fibers analyzed differs per parameter and is indicated in the figures.

Maximal tension. Maximal tension was significantly lower in type 2X (113 ± 6 vs. 95 ± 5 mN/mm2, P < 0.05) and 2B fibers (140 ± 6 vs. 108 ± 10 mN/mm2, P < 0.01) of PH rats. No significant difference in maximal tension was found in type
observed in type 2X fibers of PH rats. The MHC content per half-sarcomere reflects the number of cross bridges in parallel and was determined from MHC concentration and half-sarcomeric volume. No significant changes were observed in MHC content per half-sarcomere between PH and control diaphragm for all fiber types (Fig. 4B), suggesting that the increase in MHC concentration in 2X fibers of PH rats is caused by a reduction in fiber CSA rather than by changes in MHC content. Maximal force per half-sarcomere MHC content provides an estimation of the average force generated per cross bridge. Maximal force per half-sarcomere MHC content was significantly reduced in type 2X fibers of PH rats (Fig. 4C, P < 0.001). A similar reduction in force per half-sarcomere MHC content was found in type 2B fibers of PH rats, but this did not reach significance due to the low number of fibers analyzed (P = 0.086).

Cross bridge cycling kinetics. To determine whether the reduced maximal tension is caused by changes in cross bridge cycling kinetics, the rate of force redevelopment (k_f) and tension cost were determined. For a typical force recording during the tr protocol, see Fig. 3A. As expected, the k_f of type 2X and 2B fibers was significantly higher than in type I and 2A fibers in both groups (Fig. 3B). However, the k_f was not significantly different in any of the fiber types between control and PH rats. ATP consumption rate during isometric contraction was determined in a total of 41 diaphragm muscle bundles of control and PH rats. Note that diaphragm fiber bundles were used because the volume of one single fiber was not large enough to accurately measure ATP consumption. For a typical recording of the simultaneous measurement of ATP consumption and tension, see Fig. 3C. Tension cost of muscle contraction was calculated by normalizing ATP consumption rate to maximal tension. As can be seen in Fig. 3D, tension cost was not significantly different between control and PH rat diaphragm muscle. The fiber type composition of the fiber bundles used for tension cost measurements was analyzed. PH diaphragm bundles had significantly more type 2A fibers and a tendency toward fewer type 2B fibers (see Table 1). Both k_f and tension cost were not different, thus suggesting that changes in cross bridge cycling kinetics are not the cause of the reduced maximal tension.

Myosin heavy chain concentration. To evaluate whether changes in MHC concentration contribute to the reduced maximal tension, the MHC concentration was determined in each muscle fiber used for the contractile measurements. As shown in Fig. 4A, a significant increase in MHC concentration was observed in type 2X fibers of PH rats. The MHC content per half-sarcomere reflects the number of cross bridges in parallel and was determined from MHC concentration and half-sarcomeric volume. No significant changes were observed in MHC content per half-sarcomere between PH and control diaphragm for all fiber types (Fig. 4B), suggesting that the increase in MHC concentration in 2X fibers of PH rats is caused by a reduction in fiber CSA rather than by changes in MHC content. Maximal force per half-sarcomere MHC content provides an estimation of the average force generated per cross bridge. Maximal force per half-sarcomere MHC content was significantly reduced in type 2X fibers of PH rats (Fig. 4C, P < 0.001). A similar reduction in force per half-sarcomere MHC content was found in type 2B fibers of PH rats, but this did not reach significance due to the low number of fibers analyzed (P = 0.086).

Ca²⁺ sensitivity of force generation. To study the Ca²⁺ sensitivity of force generation in diaphragm muscle fibers, the fibers were exposed to solutions with increasing Ca²⁺ concentrations. The tension-pCa relation of type 2X fibers of control and PH rats show a significant reduction in tension starting at a pCa of 5.8 (Fig. 5A). When generated force was normalized to maximal force, a significant rightward shift of the normalized tension-pCa relation in type 2X fibers was observed (Fig. 5B), indicating reduced Ca²⁺ sensitivity. The Ca²⁺ concentration at which 50% of maximal tension is reached (pCa₅₀) was determined in all fibers. A significant reduction of pCa₅₀ in type 2X fibers of PH rats was found (5.78 ± 0.02 vs. 5.67 ± 0.03, P < 0.05), whereas no changes were observed in type I and 2B fibers. The steepness of the tension-pCa curve (n_Hill) is a measure of the cooperativity of Ca²⁺ activation. In type 2X fibers, n_Hill was significantly reduced in PH rats (3.23 ± 0.28 vs. 2.03 ± 0.20, P < 0.05).

Oxidative Stress

As indicated by de Man et al. (8), PH rats show upregulation of the ubiquitin-proteasome pathway in the diaphragm muscle. A possible trigger for the upregulation of this pathway may be nitrosative/oxidative stress due to the increased muscle activity. To investigate whether nitrosative/oxidative stress is increased in the diaphragm muscle of PH rats, we measured nitrotyrosylation and carbonylation of diaphragm muscle proteins.

Nitrotyrosylation. Peroxynitrite formation was evaluated by detection of nitrotyrosine. Monoclonal antinitrotyrosine antibody detected several protein bands between molecular masses of 36 and 130 kDa in both PH and control diaphragm muscle (see Fig. 6A). All protein bands observed were present in both control and PH diaphragm muscle. There was a strong trend (P = 0.055) toward lower total nitrotyrosine formation in PH diaphragm muscle compared with control. A significant lower nitrotyrosine optical density level was found in PH diaphragm muscle at a protein band around 30 kDa (P = 0.002, Fig. 6A).

Carbonylation. Protein carbonylation was determined by an Oxyblot procedure in which carbonyls react with DNP, which produces the corresponding hydrazone; the oxidized protein can then be detected with Western blot analysis with an anti-DNP antibody. Several protein bands between molecular masses of 35 and 250 kDa in both PH and control diaphragm muscles were detected (Fig. 6B). Total oxyblot optical density levels were not different between control and PH diaphragm.
muscle, and there was also no change in optical density of specific protein bands.

**EDL Single Muscle Fiber Contractility**

The observed changes in the diaphragm of PH rats were predominant in fast muscle fibers. Therefore, to evaluate whether these changes are specific to the diaphragm and are not part of a generalized muscle weakness, we determined the contractile properties of single fibers of the fast-twitch non-respiratory EDL muscle. In total, 54 fibers from the EDL of control and PH rats were used for contractile measurements. Fibers that coexpressed different isoform (2X/2B) were excluded for further analysis. No significant changes in maximal tension in 2B (182 ± 5 mN/mm², control vs. PH) and 2X fibers (123 ± 5 vs. 128 ± 22 mN/mm², control vs. PH) were observed (Fig. 7A). There was also no change in Ca²⁺ sensitivity (2B: 5.80 ± 0.02 vs. 5.83 ± 0.02, 2X: 5.75 ± 0.02 vs. 5.68 ± 0.08) in both fiber types (Fig. 7B).

**DISCUSSION**

This is the first study to investigate the effects of PH on single skinned diaphragm muscle fiber function. The data show that, in PH rats, type 2X and 2B fibers of the diaphragm produce less maximal tension. This weakness of fast (type 2) diaphragm fibers is, at least partly, caused by a reduction of force generated per cross bridge. In addition to the reduced maximal tension, Ca²⁺ sensitivity of force generation is significantly lower in type 2X PH diaphragm fibers, which could contribute to diaphragm muscle weakness at physiological calcium concentrations. These findings strongly suggest that sarcomere function is impaired in the diaphragm of PH rats. Interestingly, no changes in maximal tension and Ca²⁺ sensitivity were observed in the EDL muscle. This indicates that weakening of diaphragm muscle fibers in PH rats is a specific local process and is not part of a general systemic muscle weakness.
Reduced Maximal Tension in Diaphragm Muscle

Recently, a reduction of twitch and tetanic force generation in diaphragm muscle strips of PH rats has been reported by De Man et al. (8). To investigate whether this decreased force generation is caused by changes at the level of the sarcomere, the smallest contractile unit of muscle, we studied the contractile performance of skinned diaphragm fibers. In demembranized (skinned) fibers, the membranous structures are made permeable while leaving the sarcomeres intact. By exposing these cells to exogenous calcium, sarcomere function could be studied without confounding effects of, for instance, the sarcoplasmic reticulum or mitochondria. In these preparations, a significant decrease in the maximum force-generating capacity of type 2 (fast-twitch) diaphragm fibers in PH rats was observed. Thus these findings strongly suggest that sarcomere function is impaired in PH rats.

We next aimed to unravel the mechanisms that underlie sarcomeric weakness in PH. The active force generated by sarcomeres in single skinned fibers is determined by strongly bound cross bridges. During the cross bridge cycle, unbound nonforce-generating cross bridges move to an actin-bound force-generating state followed by ATP-driven cross bridge release back to the nonforce-generating state (3, 11). The transition between the force- and nonforce-generating states of the cross bridge can be described by two rate constants, one for cross bridge attachment ($k_{app}$) and one for cross bridge detachment ($k_{diss}$) (3). These two rate constants determine the fraction of force-generating cross bridges during activation ($\alpha_\text{fs}$), and a change in one or both will affect this fraction and thus force production (Eq. 4). In addition to the fraction of force-generating cross bridges, force production in skinned fibers is determined by the number of available cross bridges, fraction of force-generating cross bridges, and sensitivity, expressed as $p_{Ca50}$ in type I, 2X, and 2B diaphragm muscle fibers from the diaphragm of control (□) and PH rats (■). At higher $Ca^{2+}$ concentrations, starting at $p_{Ca}$ 5.8, a significant reduction in tension was observed. $B$: normalized to maximal tension-$p_{Ca}$ relation of PH fibers. A significant rightward shift of the normalized tension-$p_{Ca}$ relation was observed in type 2X PH fibers. $C$: $Ca^{2+}$ sensitivity, expressed as $p_{Ca50}$ in type I, 2X, and 2B diaphragm muscle fibers of control (open bars) and PH rats (solid bars). $P_{Ca50}$ in type 2X fibers was significantly lower in PH rats compared with control. Numbers above bars represent the number of single fibers analyzed. Data are presented as means ± SE, *$P < 0.05$ vs. control rats.

Fig. 4: Example of an acrylamide gel. The first 5 lanes are myosin standards, lane 6 is a rat diaphragm homogenate, and lanes 7 through 11 are single diaphragm fibers of control (open bars) and PH rats (solid bars). MHC concentration was higher in type 2X diaphragm fibers of PH rats.

Fig. 5: A: tension in response to increasing $Ca^{2+}$ concentration (tension-$p_{Ca}$ relation) of type 2X fibers from the diaphragm of control (□) and PH rats (■). At higher $Ca^{2+}$ concentrations, starting at $p_{Ca}$ 5.8, a significant reduction in tension was observed. $B$: normalized to maximal tension-$p_{Ca}$ relation of PH fibers. A significant rightward shift of the normalized tension-$p_{Ca}$ relation was observed in type 2X PH fibers. $C$: $Ca^{2+}$ sensitivity, expressed as $p_{Ca50}$ in type I, 2X, and 2B diaphragm muscle fibers of control (open bars) and PH rats (solid bars). $p_{Ca50}$ in type 2X fibers was significantly lower in PH rats compared with control. Numbers above bars represent the number of single fibers analyzed. Data are presented as means ± SE, *$P < 0.05$ vs. control rats.
bridges in parallel per half-sarcomere (n), and the force per cross bridge (Fcb) (Eq. 5). Thus a decrease in maximal force should be accompanied by a change in either one or more of these three determinants (3, 11).

\[
\alpha_f = \frac{f_{app}}{f_{app} + g_{app}} = \frac{f_{app}}{k_{tr}}
\]

\[
F_{abs} = \alpha_f \cdot n \cdot F_{cb}
\]

To estimate \(\alpha_f\), we determined \(k_{tr}\), which equals \(f_{app} + g_{app}\), as well as tension cost, which is proportional to \(g_{app}\). As shown in Fig. 3, B and D, skinned fibers of PH rats showed no difference in \(k_{tr}\) (\(f_{app} + g_{app}\)) or in tension cost (\(g_{app}\)), suggesting that a change in the fraction of force-generating cross bridges (\(\alpha_f\)) does not account for the decreased maximal force generation of diaphragm fibers. However, it should be noted that the tension cost measurements were performed on skinned muscle bundles. Analysis of fiber type distribution in these bundles showed that those from PH diaphragm had significantly more type 2A fibers and also a tendency toward fewer type 2B fibers (see Table 1). Because tension cost (\(g_{app}\)) is known to be higher in 2B compared with 2A fibers (2, 13), together with the observation that PH diaphragm bundles consisted of a higher proportion of 2A fibers, the tension cost (\(g_{app}\)) per fiber type might be higher in the diaphragm bundles of PH rats. The similar \(k_{tr}\) in PH and control fibers, together with the notion that \(g_{app}\) is increased in PH fibers, indicates that \(f_{app}\) is decreased, along with \(\alpha_f\) as well. Thus a decrease in the fraction of force-generating cross bridges (\(\alpha_f\)) might partly explain the decreased maximal force generation.

To estimate the number of available cross bridges per half-sarcomere (n in Eq. 5), the MHC content per half-sarcomere was determined. Because no change in HMC content per half-sarcomere (Fig. 4B) was observed, we propose that a change in the number of available cross bridges (n) does not contribute to the reduced maximal force generation. However, in diaphragm muscle fibers from PH rats, a large decrease in the force generated per half-sarcomeric MHC content was observed (Fig. 4C), indicating that the force per cross bridge (Fcb) is reduced. The magni- tude of this reduction (type 2X: 46% reduction) suggests that this might be the main factor that causes the reduction in maximal force generation in diaphragm muscle fibers from PH rats.

In vivo, the diaphragm is not maximally activated during breathing but rather is activated at submaximal firing rates. Thus submaximal parameters of muscle function provide relevant information. The rightward shift of the normalized force-pCa curve indicates that, at a certain Ca\(^{2+}\) concentration, a smaller percentage of maximal force is generated in diaphragm fibers of PH rats compared with controls (Fig. 5). Thus the Ca\(^{2+}\) sensitivity of force generation is reduced, which implies that force generation is even more affected at submaximal than at maximal activation. These findings indicate that the previously observed rightward shift of the force-frequency curve in diaphragm muscle strips of PH rats (8) is at least partly caused by a reduction of the Ca\(^{2+}\) sensitivity of force generation.

Fig. 6. Representative examples of a nitrotyrosine Western blot (A) and an Oxyblot (B) of diaphragm muscle homogenates of a subset of rats (5 CON, open bars; 5 PH, solid bars). A: several tyrosine-nitrated proteins were detected. In PH diaphragm muscle, total nitrotyrosine optical density (OD) was lower compared with control (left, P = 0.055). A significant decrease in OD was observed in a protein band at ∼30 kDa (black box, right). B: several bands carbonylated proteins of different molecular weight (MW) were detected. Total carbonyl OD did not differ between PH and control rats. MW markers are shown in kDa. Data are presented as means ± SE, +P < 0.1 and *P < 0.05 vs. control rats.
What Might Cause Sarcomeric Dysfunction in the Diaphragm of PH Rats?

Patients with PH, as well as monocrotaline-induced PH-rats (8), have an increased breathing frequency (Fig. 1). Patients with PH hyperventilate continuously and even become hypocapnic at rest and following exercise (19, 24, 27); hypocapnia has been recognized as an independent marker of mortality in patients with PH (18). The cause of this hyperventilation is not completely understood. It might be a compensatory mechanism for increased dead-space ventilation, as has been described in left heart failure. However, the observation that both \( P_{\text{aCO}_2} \) as well as \( P_{\text{aCO}_2} \), are reduced in patients with PH (9, 18, 39) suggests a role for increased sympathetic overdrive. As a consequence of the hyperventilation, inspiratory muscle activity increases substantially. We propose that, in PH, the inspiratory muscles are not able to adapt to this increased demand and that this leads to diaphragm weakness in PH. This proposition is based on several previous studies that showed that increased diaphragm loading is associated with diaphragm fiber weakness. For instance, hyperventilation, induced by exposure to hypocapnia for 6 wk, decreased diaphragm strength and fiber size in rats, with no effects on leg muscles (20, 21). Single fiber diaphragm weakness was also found in patients with chronic obstructive pulmonary disease in whom the diaphragm is subjected to an increased load due to stiffer lungs (23, 30). Finally, diaphragm fiber weakness was observed in rats with congestive heart failure, a weakness that was associated with a reduction in MHC concentration caused by upregulation of the ubiquitin-proteasome pathway and increased proteolytic activity (17).

Both slow and fast diaphragm fibers of PH rats have been shown to have a reduced CSA (8). This diaphragm fiber atrophy was associated with increased activation of the ubiquitin-proteasome pathway, as indicated by upregulation of the E3-ligases MuRF-1 and MAFbx (8). Interestingly, the present study reveals that the decreased CSA of diaphragm fibers from PH rats is not accompanied by a decrease in MHC content, which results in a higher MHC concentration in the fibers (Fig. 4A). Despite MHC content preservation in diaphragm fibers of PH rats, the force per MHC content was lower, which suggests that the myosin present in the fibers does not function properly. It could be speculated that the dysfunction of myosin is caused by increased ubiquitination due to elevated E3 ligase expression but that it has not been degraded yet by the proteasome due to the relatively short period of the disease state; note that breathing frequency was increased only during the last 5 days before the PH rats were killed. In line with this speculation, previous work revealed that, although E3 ligase expression was elevated, proteasome activity was not increased in the diaphragm muscle of PH rats (8).

The ubiquitin-proteasome pathway can be activated by nitrosative and oxidative stress (12, 26), both of which are associated with increased muscle loading, such as occurs in the diaphragm of PH rats. However, in contrast to our expectation, nitrotyrosine formation [a widely used marker for nitrosative stress (1, 31, 34)] was decreased in the diaphragm of PH rats, whereas protein carbonylation [a marker for oxidative stress (22, 35)] remained unchanged (Fig. 6), suggesting that in PH diaphragm changes occur in the production and/or scavenging of nitric oxide rather than of reactive oxygen species. Thus, considering that neither oxidative nor nitrosative stress appears to be upregulated, we postulate that the upregulation of the ubiquitin-proteasome pathway in PH diaphragm is triggered by alternative pathways.

Conclusion

The present study shows diaphragm weakness at the single fiber level in an animal model of PH. This weakness, which is specific for the diaphragm muscle, can be explained by a decrease of the force generated per cross bridge. Additionally, Ca\(^{2+}\) sensitivity is reduced in the diaphragm fibers of PH rats and may contribute to diaphragm weakness at submaximal activation. Similar to our observations in rats with PH, pilot data from biopsies of two patients with PH revealed a substantial reduction in maximal tension in diaphragm fibers (8). The findings of the present study and the pilot data from De Man et al. (8) provide rationale to test diaphragm-centered, therapeutic strategies to improve the quality of life of patients with PH.

GRANTS

This work was supported by a VENI grant from the Netherlands Foundation for Scientific Research (NWO) to C. Ottenheijm.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES

Diaphragm Weakness in PH


