Passive properties of the diaphragm in COPD

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Submitted 22 December 2005; accepted in final form 6 July 2006

CHRONIC OBSTRUCTIVE PULMONARY disease (COPD) is one of the leading causes of death and disability in the developed world (17, 20) and is characterized by progressive airflow limitation, which is largely irreversible (20). Because of the increased resistance to airflow, pulmonary hyperinflation can develop (18), the consequence of which is a flattening of the diaphragm (2, 13), which is essential for development of transdiaphragmatic pressure, and so the capacity of the diaphragm to generate negative intrathoracic pressures decreases in pulmonary hyperinflation (13, 27).

It is has been documented that in COPD the diaphragm undergoes an adaptation to compensate for the mechanical stresses that pulmonary hyperinflation places on it. These changes involve an alteration in the myosin heavy chain (MHC) isoform expression (14), with an increase in the proportion of type I fibers, and there is evidence of structural change also occurring within the diaphragm fibers at a subcellular level. Studies have shown that in chronic hyperinflation the resting sarcomere length in human diaphragm muscle fibers decreases (23). In emphysematous rodent models, it has been proposed that the total number of sarcomeres available in the shortened muscle fiber may be fewer than in normal controls, due to a loss in their number (4). These changes enable the muscle to shorten over the “normal” range of the force-length curve during active contraction required for normal diaphragm function, but they give no insight into changes that may occur in the passive properties of the diaphragm muscle in COPD. Passive tension, or restoring force, is the force response of a muscle at rest to stretching. Much is known about passive tension in animal skeletal muscles, including the diaphragm (9, 16, 28, 32), but largely due to the technical difficulties of obtaining diaphragm muscle samples there are few data on the passive properties of the human diaphragm in health or disease.

This study aimed to answer two principle questions: 1) how COPD affects the passive properties of human diaphragm muscle by measurement of the restoring force, and 2) how these properties relate to the recognized changes in diaphragm muscle fiber MHC isoform expression in COPD. To address these questions, we measured the restoring force of single permeabilized diaphragm fibers dissected from biopsies obtained during thoracic surgery from patients with COPD and from individuals with normal pulmonary function.

MATERIALS AND METHODS

Subjects and pulmonary function testing. Biopsy specimens were obtained from the costal diaphragm of 16 individuals: 10 from patients with COPD and 6 from controls with normal lung volumes. All individuals had thoracotomies for pulmonary nodules, under general anesthesia. Patients who had previously undergone thoracic surgery were excluded from the study. All subjects gave written, informed consent; the protocol was approved by the Brompton, Harefield and National Heart and Lung Institute Research Ethics Committee; and it conformed to the standards set by the Declaration of Helsinki.

Spirometry was obtained using the best effort measured by a digital spirometer (Micromedical, Kent, UK). Lung volumes measured by whole body plethysmography and gas transfer by a single-breath technique (CompactLab System, Jaeger, Germany) (22) were performed at the discretion of the surgical team. Reference values were taken from the 1993 European Respiratory Society statement for lung function testing, and adjusted according to ethnic origin (29).

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Preparation of muscle fibers. Full-thickness diaphragm biopsies (~15 mm × 5–8 mm) were taken from the anterior costal diaphragm during thoracic surgery. Immediately after excision, the biopsies were plunged into ice-cold skinning solution and stored on ice for transportation to the laboratory. Here the muscle biopsy block was divided into smaller samples under stereomicroscopy, and the fibers were permeabilized (“skinned”) by bathing the biopsy in a series of solutions containing increasing volumes of glycerol from 0 to 50% as previously described (31). The biopsy samples were stored in 50% glycerol solution at −20°C for up to 6 wk. The composition of the skinning solutions is listed in Table S of the online supplement.

Individual muscle fibers were prepared by forceps dissection in 0% glycerol skinning solution (5 mM ATP, Ca²⁺ free) under direct stereomicroscopic vision. Fibers that were damaged (e.g., those with evidence of sarcomere irregularities) or those excessively stretched during dissection were discarded. The ends of the chosen fibers were clamped in aluminum foil T clips as previously described (5, 7), leaving a central region of ~2–2.5 mm. The fiber/T-clip units were then transferred to a specially constructed quartz trough (30-μl capacity) on a stainless steel stage of a Zeiss microscope. Surface tension prevented the relaxing solution from escaping from the ends of the trough.

The fiber was mounted horizontally between two hooks that emerged through the ends of the trough. One hook was connected to a force transducer (AE801 sensor element, Memscap, Horten, Norway) and the other to a micromanipulator. Adjustment of the micromanipulator allowed controlled stretching of the muscle fiber and the restoring force generated was transmitted through the force transducer, via an amplifier, to a millivolt meter. The signal was also displayed on an oscilloscope for analysis of restoring force-time data.

Measurement of fiber dimensions. Once suspended in the quartz trough, the fiber was illuminated with a green light-emitting diode monochromatic light source to prevent chromatic aberration, and the fiber dimensions measured through a compound microscope with a Zeiss ×40, 0.75-numerical aperture water-immersion objective. Fiber length was determined by measuring the inter T-clip distance in millimeters. Fiber width and sarcomere length were determined by digital image acquisition of the fiber using a JAI Progressive Scan CVM4 charge-coupled device camera, and the image was displayed on a computer running Image-Pro and a custom MATLAB program. This program allowed a digital image of the muscle fiber to be stored and conversion of this image from computer pixels to length in micrometers enabled accurate measurements of the muscle fiber dimensions. The process of image acquisition and processing is as follows. Images of sarcomeres were transformed into a one-dimensional distribution of pixel intensity along the fiber length, and sarcomere length was computed by fast Fourier transform (Fig. 1). The width of the fiber was also measured from the charge-coupled device image.

Single-fiber passive tension measurements. Each relaxed fiber underwent a series of progressive stretches in small increments at room temperature. On average, seven increments were applied in a range from a starting sarcomere length of 1.5–4.1 μm. The fiber was not released to its initial slack length between stretches, and after each stepwise increase in length, the inter T-clip distance was held constant for at least 60 s before recording the output from the force transducer. After the 60-s interval, the microscope camera captured the image of the fiber for analysis of sarcomere length and fiber width, using the methods described above. This time interval allowed the restoring force to reach a peak and decay to a relatively constant plateau as described previously (15, 32). In pilot studies within this laboratory, 60 s were sufficient to allow plateau tension to be achieved (Fig. 2). The output of the force transducer in millivolts was converted to newtons.

To standardize the restoring force of the muscle fibers from every sample, the data were expressed as force per cross-sectional area (CSA; kN/m²). Fiber CSA was determined assuming permeabilized fibers hold a cylindrical cross section, and the CSA was determined at a standard sarcomere length of 2.4 μm for all fibers. The force values were pooled into sarcomere length bins of 0.1 μm. Each resulting data point presented graphically represents a mean value for all the restoring forces in that sarcomere-length bin and is shown with an error bar of one SE.

![Fig. 1. Digital image acquisition of permeabilized muscle fibers. FFT, fast Fourier transform.](http://jap.physiology.org/)
Parameterization of passive tension. To compare passive tension between fibers, a quadratic was fitted to the experimental force vs. sarcomere length data for all fibers, and also for the data after combining into patient and control groups and into groups distinguished by fiber type, following the method of Prado et al. (28). Excel Solver (Microsoft) was used to calculate the best fit of the data to a quadratic $y = ax^2 + bx + c$ by minimizing the sum of the squared differences between the data and fitted value. For each fit, the area under the curve (AUC) was calculated from the integrated formula $y = \int_a^b f(x) \, dx$, where $y$ represents the sarcomere length and $y$ the corresponding passive tension.

MHC isoform determination. After mechanical experiments, a proportion of the muscle fibers tested were retained for determination of MHC isoform expression. Each single muscle fiber was placed in a cryovial containing 20 µl sample buffer (80 mM Tris-HCl, 2.3% SDS, 5% β-mercaptoethanol, 10 mM dithiothreitol, 13.6% sucrose, 0.01% bromophenol blue, 0.1% bromoethane sulfonate fluoride, 2 µM leupeptin, 1 µM pepstatin, and 12.5% glycerol) and stored at $-80^\circ$C for up to 2 mo.

SDS-PAGE was performed on 7.5% resolving gels according to the method of O’Connell et al. (21); the samples were thawed and vortexed thoroughly before being boiled for 5 min, and each well was loaded with 5 µl of sample and 5 µl of sample buffer. MHC markers consisting of single rabbit psoas and soleus fibers (muscles containing almost pure IIX and I isoforms, respectively) were then loaded in the end and middle lanes. Electrophoresis was carried out in the Hoefer SE 250 minigel system (Hoefer Scientific Instruments, San Francisco, CA), at 100 V (constant voltage) for 32 h at 4°C. After electrophoresis, the gels were silver stained and scanned (Ettan Sample gridding kit; Amersham Biosciences, San Francisco, CA) while suspended in 300 µl sample buffer. Samples were then submerged in boiling water for 5 min and vortexed.

Samples of varying concentrations (1:40–1:100) were loaded in lanes on a 1% agarose resolving gel (1% wt/vol Sea Kem Gold agarose; Cambrex Bio Science, Wokingham, UK) (30% vol/vol glyceral, 50 mM Tris-base, 0.384 M glycine, and 0.1% wt/vol SDS) cast on top of a 1-cm-high acrylamide plug (12.8% T acrylamide, 10% vol/vol glyceral, 0.5 M Tris-HCl, pH 9.3, 0.028% wt/vol ammonium persulfate, and 0.152% vol/vol N,N,N’,N’-tetramethylethylenediamine) in a Hoefer SE 600 gel unit. Gels, cooled to 8°C with a circulating water bath, were run for 5 h at 15-mA constant current. After this, the gels were silver stained and scanned as above.

Statistical analysis. Statistical analysis was performed by using Statview software (Statview, SAS Institute, Cary, NC) running on a Hewlett-Packard Compaq computer. Differences were considered significant when the $P$ value was $\leq0.05$.

RESULTS

Characteristics of the subjects. Subjects individual anthropometric and pulmonary function data are shown in Table 1. Lung volume and gas transfer measurements were performed in nine of the COPD patients and four of the control subjects. The two groups were well matched for age, height, and weight ($P > 0.05$ for all 3 variables). The COPD group had a significantly lower mean percent predicted forced expiratory volume in 1 s (FEV$_1$) ($P = 0.02$) and a significantly lower

### Table 1. Baseline anthropometric and pulmonary function data

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<th>Subject No.</th>
<th>Age, yrs</th>
<th>Height, cm</th>
<th>Weight, kg</th>
<th>BMI, kg/m$^2$</th>
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<th>FEV$_1$, %predicted</th>
<th>FVC, liters</th>
<th>FVC, %predicted</th>
<th>FEV$_1$/FVC</th>
<th>TLC, %predicted</th>
<th>RV, %predicted</th>
<th>FRC, %predicted</th>
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<th>FRC/TLC</th>
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Values are expressed as means ± SE. BMI, body mass index; FEV$_1$, forced expiratory volume in 1 s; FVC, forced vital capacity; TLC, total lung capacity; RV, residual volume; FRC, functional residual capacity; FEV$_1$/FVC, FEV$_1$-to-FVC ratio; RV/TLC, RV-to-TLC ratio; TLCO$_c$, carbon monoxide gas transfer corrected for hemoglobin. P1–P10, chronic obstructive pulmonary disease (COPD) patients; C1–C6, non-COPD controls 1–6.
mean FEV$_1$-to-forced vital capacity (FVC) ratio (FEV$_1$/FVC) ($P = 0.003$) compared with controls. As shown in Table 1, COPD patients exhibited hyperinflation defined by raised mean percent predicted total lung capacity (TLC), functional residual capacity (FRC), and residual volume (RV) compared with the control group ($P < 0.05$ for all variables), and air trapping determined by a mean RV-to-TLC ratio (RV/TLC) >50%.

**Diaphragm muscle restoring force data.** A total of 68 costal diaphragm fibers was analyzed, 44 from the hyperinflated COPD patients and 24 from controls. Figure 3 shows the restoring force response to sarcomere lengthening from all 68 muscle fibers. Second-order polynomials (quadratic models) were calculated to fit the pooled data points and the area under the curve (AUC) for each patient group over a sarcomere length range of 2.0 – 4.0 μm.

The bar chart illustrated in Fig. 4 shows the mean AUC for both COPD and controls over this range of sarcomere lengths. Comparison of the AUCs for each group with an unpaired t-test shows that the COPD group has a lower AUC than the controls ($P < 0.0001$), indicating that the passive tension is lower in COPD compared with controls for all fiber types.

**Passive tension and MHC isoforms in human diaphragm muscle.** Figure 5 gives an example of the gel electrophoresis results for homogenized diaphragm muscle samples. As can be seen in this image, diaphragm muscle from COPD patients have proportionately denser bands corresponding to type I MHC compared with the non-COPD controls, who have a denser type II MHC band. Figure 6 shows how the passive tension for individual fiber types changes between the COPD and control groups. Although in a small selection of patients, this illustration suggests that, when considering individual muscle fiber types, the restoring force in COPD patients is lower than in the non-COPD controls regardless of MHC expression.

**Titin expression in human diaphragm muscle.** Titin expression in diaphragm homogenates was analyzed in six of the COPD patients and compared with homogenates from two of the control patients. An image of these gels is illustrated in Fig. 7. The results indicate a slower migration of the titin band in COPD patients compared with the non-COPD controls.

**DISCUSSION**

This study investigating the passive properties of the human diaphragm muscle in COPD shows that when stretched the passive tension of human diaphragm muscle fibers, judged by the area under the sarcomere length-restoring force curve, is significantly lower in patients with COPD than in the diaphragm of normal control subjects. Furthermore, we show this to be the case in COPD both for type I and type II muscle fibers, so the reduction in passive tension of the diaphragm in COPD seems to occur irrespective of the change in muscle fiber MHC expression that is associated with the disease.

It is widely accepted that the principle component of muscle passive tension is the accessory protein, titin (1, 12). Titin is a
giant protein with a molecular mass of >3,000 kDa whose molecules span from the Z disk to the M line of the muscle sarcomere (11), and it is abundant in skeletal muscle cells. In animal models, it has been shown that muscle cells can modulate their stiffness by selective expression of different isoforms of titin (9, 15, 32), but the evidence as to whether the skeletal muscle of human diaphragm can modulate its titin expression into more than one isoform under different conditions is scarce. Using SDS-agarose gels, we have demonstrated that there is a difference in the titin molecule in the diaphragm muscle of COPD patients compared with non-COPD control patients, seen as a difference in the migration of titin bands between the two groups (Fig. 7). This alteration in the titin would certainly account for the lower passive tension we have found in the diaphragm in COPD. Evidence for this conclusion was greatly strengthened whilst preparing this manuscript for submission, following the publication of work by Ottenheijm et al. (25), which also reported a reduction in the passive-tension generation of diaphragm single fibers in COPD and slower migration of titin on SDS-agarose gels in COPD diaphragm compared with controls. Although Ottenheijm et al. demonstrated that the content of titin in COPD diaphragm was not different to non-COPD diaphragm, they found that there was an upregulation of gene expression in the titin molecule and postulated that this might be due to alternative splicing of the titin gene, resulting in a longer molecule. This ability of the titin molecule to lengthen, specifically through alternative splicing in the proline, glutamate, valine, and lysine (PEVK) region of the gene, has been previously demonstrated by Sarkar et al. (30). The results of the present study, therefore, provide an important independent confirmation of Ottenheijm et al.’s (25) findings specifically that, when stretched, the reduction in passive tension generated in COPD diaphragm probably occurs through an elongation of the titin molecule. In addition, the data presented in this study includes patients with more severe COPD (mean FEV1 63.3% predicted compared with 76% for Ottenheijm et al.) and also lung volume data, both of which provide a stronger link between the reduced passive tension of the diaphragm and the severity of COPD.

Recently, Prado et al. (28) suggested in animal work that the same titin isoform expression must be preserved in all fibers of the same muscle. In the present study, we have demonstrated that passive tension generation in COPD diaphragms is lower than in non-COPD controls for both type I and type II fibers (Fig. 6). As we have also illustrated that the titin molecule appears to be different in COPD, then it would seem that the suggestion of Prado et al. in their animal work also applies to human skeletal muscle and that the alteration in the titin molecule conveys a reduction in the passive properties of all diaphragm muscle fiber types in COPD.

**Determination of COPD criteria.** To distinguish between individuals with COPD and those with normal lung function, our classification of patients and controls was determined using standard clinical and spirometric criteria. The individuals recruited to this study were placed into COPD patient or control groups based on these measurements, but there is some abutment of the patient groups as is shown in Table 1. One of the patients (P1) had an FEV1 of 109% predicted but was classified as having COPD because of the presence of pulmonary hyperinflation: FRC of 121% predicted with a RV/TLC of 47% and a history of heavy smoking (57 pack/yr). One of the controls (C2) had an FEV1 of 63% predicted but was classified as a control because of the absence of hyperinflation (FRC 101% predicted). The problem with the classification of these patients arises due to the difficulty in obtaining individuals who are fit enough for thoracotomy and yet have severe COPD, especially in our own institution where lung volume reduction is often done by video-assisted thoracoscopic surgery (6) or bronchoscopically (8), both of which preclude diaphragm biopsy. Despite this there remains a significant difference between the two cohorts in the mean indexes of airflow obstruction according to the Global Initiative for Chronic Obstructive Lung Disease criteria (percent predicted FEV1 and FEV1/FVC) and in markers of pulmonary hyperinflation (percent predicted FRC, RV, TLC, and RV/TLC) (3, 26). In addition, SDS-PAGE techniques have shown that COPD fibers have proportionately more type I MHC than the controls, as indicated by the band densities in Fig. 5. These observations, plus the significant differences observed in passive tension between the COPD and control groups, suggest that our cohorts were sufficiently different.

**Analysis of passive tension.** The restoring force-sarcomere length data (Fig. 3) shows that the elasticity of the muscle fibers in the diaphragm is not linear either in COPD or in non-COPD controls. We have chosen to analyze these results using similar methods to Prado et al. (28) by applying a quadratic model to describe the force-length relationship, which fits the data well. This quadratic model is more consistent with the evidence available in the literature, where a roughly parabolic relationship is commonly described (16, 19, 28, 34), compared with the linear relationship Ottenheijm et al. (25) used in their recent paper. This method for analysis has allowed us to extract a single parameter, the AUC, from the length-tension relationship, which enables us to directly compare the passive tension properties of the muscle fibers between and within the patient groups.

**Role of extracellular matrix.** Finally, we must consider the role of the extracellular matrix as a component of passive tension in a muscle. Certainly these structures (such as collagen) have a role in the passive tension of the whole muscle, but Prado et al. (28) have demonstrated that there is little correlation between the whole muscle passive tension and the titin-borne passive tension. We have taken this discrepancy into account, by our method of single muscle fiber preparation with skinning solution that removes the extracellular matrix. Although, in vivo, these structures may be important in the passive tension of the whole muscle, we believe that our in vitro results demonstrated in this paper are solely as a result of

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**Fig. 7. Titin expression in diaphragm homogenates** Representative single vertical SDS-agarose gel of diaphragm homogenates from 2 COPD patients and 2 control (Ctrl) patients. A rabbit marker is included for reference. Arrows at the border of the image represent in ascending order: rabbit psoas titin marker band, control titin band, and COPD titin band.
the titin molecule. Preliminary experiments with collagenase treatment of permeabilized muscle fibers of rabbit skeletal muscle indicate that residual extracellular collagen does not contribute to the observed passive tension.

Role of titin in the diaphragm in COPD. Titin conveys a structural integrity within the muscle fibers, and the shorter stiffer titin in fast-twitch muscle fibers prevents sarcromere dislocation during fast contraction (10). This stiffer titin would result in a higher passive tension when stretched. Conversely, in COPD the muscle fibers of the diaphragm are predominantly slow acting, and so the need for a stiff, short titin may be diminished. As a result, the titin molecules are longer resulting in a lower passive tension. Our findings may, therefore, simply be a consequence of the change in fiber type seen in the diaphragm muscle in COPD. Indeed this may, in part, account for the sarcromere dislocation during fast contraction (10). This stiffer titin would contribute to the observed passive tension.

In conclusion, this study demonstrates that the reduced passive tension generation of the diaphragm observed in COPD arises from a compensatory adaptation of the titin molecule within the sarcromeres of the diaphragmatic muscle fibers. It has been suggested that this adaptation in the properties of the titin molecule arises from the expression of a longer more extensible titin isoform associated with the predominance of slow-twitch muscle fibers within the diaphragm in COPD, and this study provides important confirmatory evidence to support this.

ACKNOWLEDGMENTS
The authors thank Dr. T. West (Imperial College London) for help and advice with experiments.

GRANTS
This study was funded by the Medical Research Council, the National Heart and Lung Institute, and DMETA.

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