Titin and Diaphragm Dysfunction in Chronic Obstructive Pulmonary Disease

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Rationale: Recently, we have shown that Ca^{2+} -activated force generation in diaphragm single fibers is impaired in patients with mild to moderate chronic obstructive pulmonary disease (COPD). For optimal active-force generation, the passive elasticity provided by titin is indispensable.

Objectives: In the present study, we determined the passive-tension-length relations of single fibers of patients with mild to moderate COPD, hypothesizing that passive-elastic properties of diaphragm fibers are compromised.

Methods: Passive-tension-length relations were determined in diaphragm fibers from patients with and without COPD (predicted mean FEV_1 , 76 and 102%, respectively). In diaphragm homogenates titin expression was studied at the protein level by gel electrophoresis and at the transcript level by using a novel titin exon microarray.

Results: Diaphragm fibers from patients with COPD generate less passive tension on stretch. Titin content in the diaphragm did not differ between patients with and without COPD. However, titin exon transcript studies revealed up-regulation of seven exons, which code for spring elements in the elastic segment rich in proline, glutamate, valine, and lysine. Immunofluorescence analysis indicated elevated protein expression of the up-regulated splice variant in the COPD diaphragm. Simulation studies on titin molecules including the amino acids encoded by the seven up-regulated exons predicted reduced passive-tension generation on molecule stretch. **Conclusions:** Passive-tension generation of diaphragm single fibers is reduced in patients with COPD. Our results suggest that alternative splicing of the titin gene, resulting in increased length of the elastic segment rich in proline, glutamate, valine, and lysine, is involved. Interestingly, these changes occur already in patients with mild to moderate COPD.

Keywords: chronic obstructive pulmonary disease; diaphragm; single-fiber stiffness; titin; transcript studies

Dysfunction of the respiratory muscles frequently occurs in patients with severe chronic obstructive pulmonary disease (COPD) (1). As hypercapnic respiratory failure due to inspiratory muscle weakness (2) is the most important cause of death in these patients (3), understanding the underlying mechanisms of respiratory muscle dysfunction is of major clinical importance.

The diaphragm is the most important inspiratory muscle. Several studies have shown adaptations in diaphragm morphology and function in patients with COPD (4–8). For instance, we have shown that calcium-activated force generation in diaphragm single fibers is impaired in mild to moderate COPD as defined by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) I/II (8). For optimal active-force generation, passive-elastic structures are indispensable (9). However, passive elasticity in diaphragm single fibers in COPD has not been investigated.

It is generally accepted that the giant protein titin is the most important determinant of the passive-elastic characteristics of striated muscle (*see reviews by Tskhovrebova and Trinick [10] and Granzier and Labeit [11]*). Titin spans the half-sarcomeric distance from the Z-line to the M-line, thus forming a third sarcomeric filament, apart from the thick (mostly myosin) and thin (mostly actin) filaments (12). In the I-band region, titin is extensible and functions as a molecular spring that develops passive tension upon stretch. In skeletal muscle, an important source of titin's elasticity is the segment rich in proline-glutamate-valine-lysine (PEVK) residues. In the A-band, titin is inextensible due to its strong interaction with the thick filament (13, 14). The passive-elastic properties of titin are essential for maintaining structural and mechanical stability of the sarcomere during calcium activation; this stability is achieved by the maintenance of thick filaments in a central position in the sarcomere (9, 15). Recently, stiffness of titin has been shown to be calcium regulated, due to calcium binding to glutamate-rich exons located in the PEVK region of the molecule, rendering titin a calcium-dependent molecular spring that adapts to the physiological state of the cell (16). Titin has a multitude of functions that go beyond a pure mechanical role. In particular, titin functions as a stretch sensor with titin-based stiffness-regulating muscle remodeling and gene expression (17) (for a recent review, *see Reference 18*).

Titin is encoded by a single gene, which, in humans, contains 363 exons. The extensible PEVK region contains 116 exons; most code for conserved approximately 28-residue PEVK repeats and others for more complex glutamate-rich motifs (19). Alternative splicing of the PEVK exons has been shown to modulate the length of titin's spring segment and consequently the passive tension generated on sarcomere stretch (20). These changes in titin expression have been shown to regulate passive stiffness both during postnatal development (20) and in the adult heart (19, 21). Moreover, evidence is accumulating that alternative splicing of the PEVK region of titin plays an important role in muscle pathophysiology. We and others have found adjustments in splicing in various disease states of the heart (22–24) and that these adjustments are important in pathologic changes in chamber stiffness (23).

Considering the importance of passive muscle elasticity for muscle function and the crucial role of titin in muscle physiology and pathology, we measured in this study the passive tension-length

(Received in original form July 8, 2005; accepted in final form December 6, 2005)

Supported by an unrestricted educational grant from GlaxoSmithKline, The Netherlands; and by National Institutes of Health grant HL61497 (H.L.G.).

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 173, pp 527–534, 2006

Originally Published in Press as DOI: 10.1164/rccm.200507-1056OC on December 9, 2005
Internet address: www.atsjournals.org

relation of single fibers of patients with mild to moderate COPD, hypothesizing that passive-elastic properties of diaphragm single fibers are compromised. We studied titin expression at the protein level by sodium dodecyl sulfate (SDS)-agarose gel electrophoresis and at the transcript level by using a novel titin exon microarray. Results indicate that passive stiffness is reduced in COPD and that this can be explained by differential splicing of the titin gene.

Some of the results of these studies have been previously reported in an abstract (25).

METHODS

Subjects and Pulmonary Function Testing

Diaphragm muscle biopsies were obtained from 12 patients with COPD and 14 patients without COPD. Biopsies were obtained from the right anterior costal diaphragm during thoracotomy for lung cancer (stage T₁₋₃N₀₋₁M₀ in both groups). Patients were excluded from the study if they had more than 10% weight loss in the 6 mo before surgery. General characteristics and pulmonary function data are shown in Table 1. Informed consent was obtained from each patient, and the study was approved by the local ethics committee.

Diaphragm Biopsies

The fresh biopsy sample was divided into three parts: one part for single-fiber passive-tension studies, one part for immunofluorescence studies, and one part for protein analysis and titin exon microarray. The three parts were processed and stored as presented in the online supplement.

Single-Fiber Passive-Tension Studies

Approximately 1 h before passive-force measurement, the muscle bundle was transferred to cold (5°C) relaxing solution containing 1% Triton X-100 to permeabilize the plasma membrane. From the muscle bundle, segments (~2 mm) of single fibers were isolated. Subsequently, the fiber ends were attached to aluminum foil clips, and mounted on the single-fiber apparatus. Fibers that appeared injured (e.g., having loss of cross-striation or other irregularities) during microscopic examination ($\times 400$ magnification) were excluded.

Composition of activating and relaxing solutions is as reported previously (8) and in the online supplement. To ensure stable attachments throughout the mechanical protocol, the fiber and clip-hook attachments were first exposed to the high forces generated in maximal activating solution (pCa 4.5). The fibers were then kept in relaxing solution for 10 min, and passive-tension-length relations were determined by applying a repeated stretch-hold protocol, composed of a stretch of 10% of optimal length and 60-s hold to allow for stress relaxation (protocol adapted from Reference 13). At the end of the 60-s hold,

passive tension was recorded and normalized to fiber cross-sectional area. Additional details are given in the online supplement.

At the end of the passive-tension studies, myosin heavy-chain isoform composition of the fiber was identified by SDS-polyacrylamide gel electrophoresis as described previously (8).

SDS-Agarose Gel Electrophoresis, Transcript Studies, and Immunofluorescence Microscopy

The methods for determination of protein expression by SDS-agarose gel electrophoresis, transcript studies and simulation of force-sarcomere length relationships, and immunofluorescence studies are in the online supplement.

Statistical Methods

To evaluate the statistical significance of differences in single-fiber passive-tension data between patients with and without COPD a repeated-measures analysis was performed with *post hoc* testing for each fiber length. Differences between the COPD and non-COPD groups were analyzed with *t* tests or, for the microarray studies, for which the small number of experiments made it difficult to assess whether data values are normally distributed, with the Wilcoxon rank-sum test. Because of limited diaphragm tissue available per patient, data from the single-fiber, SDS-agarose, transcript, and immunofluorescence studies are not based on the same patients, although there is extensive overlap. A *p* value less than 0.05 was used as criterion for statistical significance.

RESULTS

Subject Characteristics

Patient characteristics and pulmonary function data are shown in Table 1. Patients with COPD included for single-fiber passive-tension, SDS-agarose gel electrophoresis, immunofluorescence, and microarray studies were classified as having mild (stage I) or moderate (stage II) COPD on the basis of GOLD classification (26, 27).

Single-Fiber Passive-Tension Studies

To measure passive tension, skinned fibers at optimal length (2.4 μ m) were stretched with 10 increments of 10% of optimal length. A typical passive-tension response to imposed length steps is illustrated in Figure E1 of the online supplement. Single fibers (*n* = 68) from seven patients with COPD and single fibers (*n* = 51) from five patients without COPD were used for passive-tension measurements. These 119 fibers expressed a single myosin heavy-chain isoform (75 myosin heavy-chain slow fibers and 44 myosin heavy-chain fast fibers). Fibers that coexpressed myosin heavy-chain slow and 2A were excluded from further analysis.

TABLE 1. PATIENT CHARACTERISTICS

	Single-Fiber Passive-Tension Studies		SDS-Agarose Studies		Microarray Studies		Immunofluorescence Microscopy	
	Non-COPD (<i>n</i> = 5)	COPD (<i>n</i> = 7)	Non-COPD (<i>n</i> = 10)	COPD (<i>n</i> = 11)	Non-COPD (<i>n</i> = 6)	COPD (<i>n</i> = 5)	Non-COPD (<i>n</i> = 10)	COPD (<i>n</i> = 9)
Male/female	5/0	6/1	8/2	9/2	5/1	5/0	7/3	8/1
Age, yr	56 $\pm$ 4	60 $\pm$ 3	60 $\pm$ 3	60 $\pm$ 2	60 $\pm$ 4	61 $\pm$ 3	55 $\pm$ 3	59 $\pm$ 2
BMI, kg/m ²	27 $\pm$ 1	24 $\pm$ 2	27 $\pm$ 1	25 $\pm$ 2	28 $\pm$ 2	27 $\pm$ 3	27 $\pm$ 2	25 $\pm$ 2
FEV ₁ , % predicted	102 $\pm$ 5	76 $\pm$ 8	92 $\pm$ 4	71 $\pm$ 7	93 $\pm$ 6	89 $\pm$ 9	93 $\pm$ 5	71 $\pm$ 5
VC, % predicted	97 $\pm$ 6	100 $\pm$ 12	84 $\pm$ 5	98 $\pm$ 9	86 $\pm$ 8	113 $\pm$ 13	91 $\pm$ 5	95 $\pm$ 7
FEV ₁ /VC, %	82 $\pm$ 3	59 $\pm$ 2	77 $\pm$ 2	54 $\pm$ 3	78 $\pm$ 3	60 $\pm$ 2	78 $\pm$ 2	57 $\pm$ 2
TLC, % predicted	100 $\pm$ 11	99 $\pm$ 3	95 $\pm$ 4	106 $\pm$ 6	96 $\pm$ 6	116 $\pm$ 10	99 $\pm$ 3	102 $\pm$ 6
DL _{CO} /VA, % predicted	89 $\pm$ 6	82 $\pm$ 7	96 $\pm$ 6	81 $\pm$ 5	97 $\pm$ 11	79 $\pm$ 11	97 $\pm$ 6	87 $\pm$ 4
PaO ₂ , kPa	12.3 $\pm$ 0.0	11.5 $\pm$ 0.4	10.9 $\pm$ 0.5	11.4 $\pm$ 0.4	11.2 $\pm$ 0.7	11.4 $\pm$ 0.5	11.6 $\pm$ 0.4	11.7 $\pm$ 0.2
PaCO ₂ , kPa	4.6 $\pm$ 0.0	4.8 $\pm$ 0.2	4.9 $\pm$ 0.1	5.2 $\pm$ 0.1	4.9 $\pm$ 0.1	4.7 $\pm$ 0.2	5.3 $\pm$ 0.2	5.3 $\pm$ 0.2

Definition of abbreviations: BMI = body mass index; COPD = chronic obstructive pulmonary disease; DL_{CO}/VA = carbon monoxide transfer coefficient per alveolar volume; SDS = sodium dodecyl sulfate.

Patient characteristics for single fiber, SDS-agarose, microarray, and immunofluorescence microscopy studies.

Values are means $\pm$ SEM.

Averaged passive-tension-length relations of slow and 2A fibers from patients with and without COPD are shown in Figures 1A and 1B. These results clearly illustrate that passive tension generated on fiber stretch was significantly lower in patients with COPD compared with patients without COPD. Type 2A fibers from patients with COPD showed fiber-length-dependent reduction of passive tension, with more pronounced tension reduction at higher fiber lengths (Table 2). This was not found in slow fibers.

SDS-Agarose Gel Electrophoresis

Titin expression was analyzed in diaphragm samples from 11 patients with and 10 patients without COPD. Overall, the electrophoresis patterns were similar in quality with clear T1 bands and barely visible (sometimes absent) T2 bands (T1 is the intact titin molecule and T2 is a major degradation product). Titin mobility tended to be slower in some patients with COPD (Figure 2A, left and middle panels) but this was not a consistent finding (Figure 2A, right panel). Using quantitative densitometry, we determined the amount of titin in the diaphragm samples. As shown in Figure 2B, total titin content did not differ in diaphragm samples from patients with and without COPD. Also, T1 (0.099 ± 0.012 vs. 0.092 ± 0.013 , arbitrary units, non-COPD vs. COPD) as well as T2 (0.013 ± 0.005 vs. 0.005 ± 0.002 , arbitrary units, non-COPD vs. COPD) content did not differ between the two groups. On the other hand, myosin heavy-chain content was lower in diaphragm samples from patients with COPD

TABLE 2. FIBER-LENGTH DEPENDENCE OF PASSIVE-FORCE REDUCTION IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE

% of Optimal Fiber Length	Passive-Force Reduction (%)	
	Slow-Type	Type 2A
110	35	23
120	42	31
130	42	38
140	42	43
150	40	45
160	40	47
170	40	46
180	40	45
190	41	43
200	38	38

Diaphragm single fibers from patients with chronic obstructive pulmonary disease show fiber-length-dependent reduction of passive-force generation in type 2A, but not slow-type, fibers.

(Figure 2C), confirming previous findings from our lab (8). The ratio of titin to myosin heavy chains did not differ significantly between the two patient groups (0.141 ± 0.02 vs. 0.151 ± 0.01 , arbitrary units, non-COPD vs. COPD). The ratio of slow to fast myosin heavy-chain isoform was higher in diaphragm samples from patients with COPD (Figure 2D), and nebulin content was lower in diaphragm samples from patients with COPD (Figure 2E).

Immunofluorescence Microscopy: T12, T51, and 9D10

Immunofluorescence studies were performed on diaphragm cryosections from nine patients with COPD and 10 patients without COPD. The cryosections were incubated with antibodies T12, T51, and 9D10, which bind to specific titin epitopes near the Z-line, the M-line, and in the PEVK region, respectively. Immunofluorescence analysis showed similar staining intensities of T12, T51, and 9D10 between patients without (Figures 3A, 3C, and 3E) and patients with COPD (Figures 3B, 3D, and 3F).

Titin Exon Microarray

We compared titin exon expression in diaphragm samples from five patients with and six without COPD (Figures 4A, 4B, and 4C). Our results show that seven exons are more than threefold up-regulated in the transcripts of patients with COPD. All up-regulated exon code for PEVK elements found in titin's elastic I-band region. The combined molecular mass encoded by these exons is approximately 33 kD. The effects of up-regulated PEVK segments on passive-tension characteristics of a single titin molecule were simulated using a serially linked, wormlike chain model. Using the wormlike chain model equation, we calculated the relative fractional extension of the tandem Ig region and the PEVK segment based on the principle of equivalence of forces within a serially linked mechanical system (for details, see online supplement). Figure 4D shows that the up-regulated exons, as observed in the diaphragms of patients with COPD, result in lower passive-tension generation upon stretch of single titin molecules. To investigate up-regulation of the alternatively spliced exon 156 at the protein level, we double-stained diaphragm cryosections with antibody T12 (against a titin epitope near the Z-line) and an antibody directed against the titin domain encoded by exon 156 (antibody X156). All diaphragm fibers from patients with COPD showed positive staining for X156 (green, Figure 5B), whereas in those from patients without COPD, this antibody stained less intensely in the majority of fibers (green, Figure 5A). As expected, all fibers from both patient groups

Figure 1. Passive-tension-length relations of slow (A) and 2A (B) diaphragm fibers from patients with (triangles) and without (squares) chronic obstructive pulmonary disease (COPD). Passive tension generated by fibers from patients with COPD was lower at all fiber lengths and in both fiber types compared with that of fibers from patients without COPD. Data are presented as mean $\pm$ SEM. * $p < 0.05$ difference from non-COPD.

Figure 2. Sodium dodecyl sulfate (SDS)-agarose electrophoresis of diaphragm homogenates from three patients with COPD and three patients without COPD. (A) SDS-agarose analysis of titin, nebulin, and myosin heavy-chain expression. Human soleus muscle was included as a titin migration reference. Titin mobility tended to be lower in some patients with COPD (left and middle panels) but not in others (right panel). (B) Densitometric quantification of protein bands revealed similar titin content in diaphragm homogenates from patients with and without COPD. Myosin heavy-chain and nebulin content were lower in diaphragm homogenates from patients with COPD (C and E, respectively). Ratio of myosin heavy-chain fast-to-slow was lower in COPD (D). * $p < 0.05$ difference from non-COPD.

stained positive for T12 (red, Figure 5C and 5D). Confocal imaging showed clear staining of T12 near the sarcomeric Z-line and X156 in the two sarcomeric I-band regions in patients with COPD (Figure 5F), with the latter being more diffuse and more dim in patients without COPD (Figure 5E).

DISCUSSION

The present study is the first to investigate passive-tension characteristics of diaphragm muscle single fibers in humans, and particularly in patients with COPD. Our results demonstrate that skinned diaphragm single fibers from these patients generate less passive tension on stretch than fibers from patients without COPD. In skinned fibers, titin is the major source of passive tension. Using SDS-agarose and immunofluorescence assays, we

Figure 3. Immunofluorescence analysis showing similar staining of T12 (specific for a titin epitope near the sarcomeric Z-line), T51 (specific for a titin epitope near the sarcomeric M-line), and 9D10 (specific for repeating epitopes in the proline-glutamate-valine-lysine rich—PEVK—region of titin) between patients without (A, C, and E) and with COPD (B, D, and F). Bar: 100 μ m.

Figure 4. (A) Analysis of diaphragm transcripts of patients with and without COPD by a 50-mer oligonucleotide array containing 385 sequence-verified probes representing all of titin's human gene exons, and normalization, positive, and negative controls. RNA isolated from diaphragm samples from patients with and without COPD was reverse transcribed, biotinylated, and hybridized to the exon microarray. (B) Example of constitutively expressed exon (5) and differentially expressed exons (156, 168, 170, 176, 178, 196, and 212) in diaphragm samples from patients with and without COPD. Two rows per sample show duplicate spots and reveal reproducibility. 5MM indicates a 50-mer from exon 5 including 5-bp mismatches as a control for hybridization stringency. (C) Bar graph showing mean and SEM values of differentially expressed exons. All exons listed, which code for the extensible PEVK segment of titin, are up-regulated by at least threefold ($p < 0.05$) in diaphragm samples from patients with COPD (black bars) compared with diaphragm samples from patients without COPD (white bars). (D) The effects of up-regulated PEVK segments on passive tension of a single titin molecule, simulated using a serially linked, wormlike chain model. The up-regulated exons in the diaphragm samples of patients with COPD result in passive-tension generation on stretch of single titin molecules.

found no significant difference in titin content in the diaphragm in the two groups. However, titin exon transcript studies revealed up-regulation of seven exons. Interestingly, all up-regulated exons code for spring elements in the elastic PEVK segment. Additional immunofluorescence studies showed higher expression at the protein level of the up-regulated exon 156 in the COPD group. These data suggest that alternative splicing of the titin gene occurs in the diaphragm of patients with COPD, resulting in increased length of the PEVK segment. Therefore, we postulate that alternative splicing of the titin gene contributes to the observed decreased passive-tension generation upon diaphragm-fiber stretch in patients with COPD. Interestingly, these changes occur already in patients with mild to moderate COPD (GOLD stage I/II).

Reduced Diaphragm Single-Fiber Passive Tension in COPD

In severe COPD, cellular alterations in the diaphragm have been shown to occur (4–6, 28). For instance, in these patients the diaphragm undergoes a fiber type shift toward more fatigue resistant fibers expressing slow-type myosin heavy chain (4). The present study shows that this shift in fiber type already occurs in mild-to-moderate COPD (Figure 2D), which confirms data from a previous study (29). Recently, we have demonstrated reduced active-force generation in diaphragm single fibers from patients with mild to moderate COPD, with concomitant loss of myosin heavy chain in those fibers (8). For optimal contractile

function, titin's passive-elastic properties are indispensable (9). Therefore, besides active-force generation, passive-force characteristics of muscle fibers provide important information on muscle function. Our data demonstrate that diaphragm fibers from patients with COPD generate less passive tension on fiber stretch than fibers from patients without COPD (Figure 1). As passive tension in these fibers is mainly determined by the properties of titin, these findings indicate that titin-related sarcomeric alterations occur in the patients with COPD.

Reduced Passive Tension: Due to Loss of Titin Content or Alternative Splicing?

First, we investigated if the loss of passive tension in diaphragm fibers of patients with COPD was associated with loss of titin content. Gel electrophoresis of diaphragm homogenates revealed a prominent titin band, T1, at the top of the gel (Figure 2A) and often also a weak T2 band. The T2 band is likely to be a proteolytic product of T1 (30). Quantitative densitometry showed that there was no significant difference in total titin content (T1 and T2) between patients with and without COPD (Figure 2B). Also, there was no difference in content of T2 titin between the two patient groups, indicating no increased titin proteolysis in the diaphragm in COPD. In line with the gel data, diaphragm cryosections from patients with and without COPD showed comparable staining for three titin antibodies: T12,

Figure 5. Immunofluorescence analysis of antibody X156 directed against the titin domain encoded by exon 156. Double-staining with antibodies X156 and T12 revealed greater staining intensity of X156 in diaphragm cryosections from patients with COPD (green, A–B), whereas staining intensity of T12 was similar in those of both patient groups (red, C–D). Bar: 50 μ m. Confocal microscopy demonstrated the expected staining pattern of both antibodies: red striations of T12 indicate the location of the sarcomeric Z-lines and green striations of X156 indicate the PEVK regions of the sarcomere (E–F). Bar: 2.5 μ m.

specific for a titin epitope near the Z-line; T51, specific for a titin epitope near the M-line; and 9D10, specific for repeating epitopes in the PEVK region of titin. According to these results, titin is preserved in the diaphragm in COPD. Consequently, the decreased passive tension in diaphragm fibers in COPD is unlikely to result from reduced titin content.

To investigate exon composition of titin we used a titin exon microarray (20) that allows evaluation of all 363 exons of the human titin gene. The transcript studies revealed greater than threefold up-regulation of seven titin exons in the diaphragm of patients with COPD. Remarkably, all the up-regulated exons code for spring elements found in the PEVK segment of titin. The results of the immunofluorescence studies with an antibody against the up-regulated PEVK exon 156 support the microarray results. This antibody stained more intensely in cryosections from patients with COPD (Figure 5). As a further test of the microarray findings, future studies with real-time polymerase chain reaction should also be conducted. As a result of the additional exons, the overall length of the PEVK segment will be longer in diaphragm fibers from patients with COPD compared with fibers from patients without COPD. In line with this, Figure 2 shows that titin from the diaphragm of patients with COPD tends to migrate slower compared with titin from patients without COPD. However, as the up-regulated exons code for approximately 33 kD (out of a total molecular mass > 3500 kD), these differences are difficult to visualize as mobility difference

in gels. We were able to detect T1 migrating slower in samples from some patients with COPD but not in those from others (Figure 2).

Using previously determined molecular characteristics (31), we simulated the force–sarcomere-length relationship of single titin molecules with the exon composition as found in the diaphragm of patients with and without COPD. As shown in Figure 4D, the up-regulated seven exons in COPD, while only coding for approximately 33 kD, decrease passive-tension generation of titin molecules. Therefore, alternative splicing of the titin gene in COPD is likely to contribute to the lower passive-tension generation of diaphragm fibers from these patients. The simulated force–sarcomere length predicts a length-dependent passive-tension reduction in COPD, with more pronounced reduction of tension at higher sarcomere lengths. Indeed, relative tension reduction was more pronounced at higher fiber lengths in type 2A fibers (see Figure 1B). In slow-type fibers (Figure 1A), no length-dependent force reduction was observed, indicating additional mechanisms to be at play.

The molecular mechanisms underlying alternative splicing of the titin gene remain unknown. Establishing if, for instance, systemic inflammation in patients with mild to moderate COPD or the increased workload of the diaphragm play a role would be of interest, but was beyond the scope of the present study.

Effects of Reduced Titin Stiffness on Diaphragm Function *In Vivo*

The described decrease in titin stiffness in the diaphragm of patients with COPD may affect *in vivo* diaphragm function in these patients via multiple pathways. First, the reduced stiffness of titin induces structural and mechanical instability of sarcomeres in the diaphragm of patients with COPD, and could result in misalignment of thick filaments in the sarcomere and inability of the muscle fiber to resist sarcomere inhomogeneity during calcium activation. Overstretching of the sarcomere might eventually be causative for the sarcomeric damage that is known to occur in the diaphragm of patients with severe COPD (32). Second, titin's stiffness has been postulated to affect myofibrillar calcium sensitivity. A study by Cazorla and colleagues strongly suggested that titin-based passive tension modulates the lattice spacing between the myosin and actin filaments in mouse heart, and that increased lattice spacing reduces calcium sensitivity of force generation (33). Through this mechanism, the reduced stiffness of titin would increase the distance between myosin and actin filaments and consequently reduce myofibrillar calcium sensitivity. Interestingly, previous work from our lab did indeed reveal decreased myofibrillar calcium sensitivity in the diaphragm of patients with COPD (8). Finally, recent studies indicate that titin plays a role in signaling pathways either directly by altering the function of the titin kinase or indirectly via influencing titin-binding proteins that control gene expression and protein turnover in response to mechanical stretch (17, 18). Although speculative, the reduced stiffness of titin in the diaphragm of patients with COPD might negatively influence production of muscle proteins and could play a role in the reduced myosin and nebulin content in the diaphragm of these patients (see Figures 2C and 2E and previous work from our lab [8]). Unfortunately, the content of proteins with lower molecular weight (< 200 kD) could not be determined in the present study due to methodologic constraints. Thus, it is possible that the modification in splicing of the titin gene impacts not only passive tension but also protein turnover, a possibility that requires future study, especially since loss of myosin is likely to be a key mechanism in the etiology of diaphragm-muscle weakness in COPD.

Decreased Nebulin Content in Diaphragm of Patients with COPD

The present study is the first to demonstrate that nebulin content is significantly reduced in the diaphragm of patients with COPD (Figure 2E). Nebulin, another giant protein (~700 kD), spans the entire length of the thin filament. Although nebulin was discovered more than two decades ago (34), it remains a somewhat nebulous component of striated muscle. It has been proposed that nebulin functions as a ruler that specifies thin filament length (35). Other studies suggest that nebulin has important functions in the sarcomere in addition to its structural roles, such as regulation of contraction by reduction of the velocity of actin's sliding over myosin and by cooperation with tropomyosin and troponins and signal transduction conducted through its phosphorylation sites (36). Therefore, loss of nebulin might further enhance the limitations in diaphragm-muscle function in patients with COPD.

In summary, the present study demonstrates reduced passive-tension generation on diaphragm-fiber stretch in COPD. Titin transcript studies reveal increased expression of exons coding for spring elements in the elastic region of titin, and immunofluorescence studies suggest increased expression of the splice variant at the protein level. We postulate that alternative splicing of the titin gene is related to the observed decreased passive-tension generation in diaphragm fibers in COPD and contributes to respiratory-muscle dysfunction in these patients. Apparently, these changes occur early in the disease process, as our patients had only mild to moderate COPD (GOLD I/II).

Conflict of Interest Statement: None of the authors have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgment: The authors thank S. van der Locht (NV Organon, The Netherlands) for technical assistance regarding myosin heavy-chain isoform determinations; they thank Paul H. K. Jap for his expertise in the field of muscle morphology; and they also thank Dr. A. Verhagen (Radboud University Nijmegen Medical Centre, The Netherlands), Dr. F. van den Elshout, Dr. S. van Sterkenburg, Dr. W. de Vries, Dr. T. Bloemen (Rijnstate Hospital Arnhem, The Netherlands), Dr. F. Smeenk, and Dr. B. van Straten (Catharina Hospital Eindhoven, The Netherlands) for collecting the diaphragm muscle biopsies.

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