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Atrophy and hypertrophy signalling in the diaphragm of patients with COPD

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ABSTRACT: We investigated whether atrophy and hypertrophy signalling were altered in the diaphragm of chronic obstructive pulmonary disease (COPD) patients.

We studied diaphragm fibre dimensions and proportion, expression of markers of the ubiquitin-proteasome pathway, nuclear factor (NF)-κB pathways, muscle regulatory factors and myostatin in diaphragm biopsies from 19 patients with severe COPD and 13 patients without COPD.

Type I proportion was significantly increased in the diaphragm of COPD patients while type II proportion was decreased. The cross-sectional area of all fibre types was reduced in the COPD patients. In addition, MAFbx mRNA was higher in the diaphragm of COPD patients while Nedd4 mRNA decreased. Cytoplasmatic levels of inhibitor protein IκBα and IκBβ were decreased in the COPD patients as was NF-κB p50 DNA-binding activity. MyoD mRNA and its nuclear protein content were decreased in the diaphragm of COPD patients and myogenin mRNA and protein levels remained unchanged. Myostatin mRNA was decreased but its protein levels in the nuclear and cytoplasmic fraction were significantly increased in the COPD patients.

These data show that the ubiquitin-proteasome pathway, the NF-κB pathway and myostatin protein were up-regulated in the diaphragm of COPD patients while MyoD expression was reduced. These alterations may contribute to diaphragm remodeling in COPD.

KEYWORDS: Diaphragm, muscle, myostatin, proteasome pathway, transcription factors

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Inspiratory muscle weakness in patients with chronic obstructive pulmonary disease (COPD) is clinically relevant, since maximum inspiratory pressure is correlated with survival in these patients. Adaptation of the diaphragm revealed a greater proportion of type I fibres with a decreased proportion of type II fibres in COPD patients [1, 2]. This shift towards a slower, more fatigue-resistant profile, which is also consistent with the adaptation observed in the expression of the SERCA (sarcoplasmic endoplasmic calcium adenosine triphosphatase) pumps [3], has been linked to chronic increased activity of the diaphragm in COPD patients [4]. Reduced force generation of single diaphragm fibres with decreased myosin content was also found in response to COPD [5]. Moreover, these fibres had decreased calcium sensitivity that could contribute to muscle weakness at submaximal activation [5]. These changes were already present in patients with mild-to-moderate COPD. Impaired cross-bridge cycling kinetics, fibre atrophy and sarcomeric injury were also observed [2, 5]. Structural changes in the titin molecule may weaken the stability of the muscle filaments, resulting in fibre damage [2, 5]. Finally, protein oxidation, increased oxidative capacity and mitochondrial function occur in line with the progression of the disease [5].

Whether atrophy and hypertrophy signalling pathways are altered in the diaphragm of COPD patients has been poorly investigated. However, this area is of particular interest knowing that COPD is the most common respiratory disease and the diaphragm the most important inspiratory muscle. The potential role of the ubiquitin-proteasome pathway, the nuclear factor (NF)-κB pathway, muscle regulatory factors (MRFs) and myostatin was investigated in the present study.

The ubiquitin-proteasome pathway is one of the major proteolytic systems involved in muscle protein breakdown. Before degradation by the proteasome, proteins need to be ubiquitinated, a process requiring an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme and an E3 ubiquitin-ligating enzyme. The latter is important as it determines which proteins are

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targeted for degradation. Among the E3 ligases, MuRF1 and MAFbx are rapidly up-regulated during muscle atrophy and are, therefore, used as markers of the ubiquitin-proteasome pathway activation. Another E3-ligase, Nedd4, was shown to be up-regulated in situations of disuse but not in catabolic states [6].

The classical NF-κB pathway is activated during the inflammatory processes. After degradation of the inhibitory protein IκB, the p65/p50 heterodimers move to the nucleus where they target genes of inflammatory pathways [7]. Interestingly, disuse muscle atrophy caused by unloading was shown to activate an alternative NF-κB pathway involving p50 and Bcl-3 but not p65 [7].

The MRFs, MyoD and myogenin, belong to the basic helix-loop-helix transcription factors. In mature skeletal muscles, MyoD is mainly expressed in fast-twitch muscles and myogenin in slow-twitch muscles [8]. Changes in MyoD and myogenin have been reported after muscle disuse caused by denervation, immobilisation or hindlimb suspension [8]. Alterations in MyoD and myogenin have been suggested to drive muscle phenotype adaptations [8].

Finally, myostatin, an inhibitor of skeletal muscle mass, has been shown to increase during atrophy caused by hindlimb unloading or microgravity, as well as in conditions of glucocorticoid-induced muscle atrophy [9]. Myostatin is believed to suppress proliferation and differentiation of muscle precursor cells and to up-regulate the ubiquitin-proteasome system [9].

The implication of these pathways in diaphragm remodeling in response to COPD is still unknown. Therefore, the expression of markers of atrophy signalling (MuRF1, MAFbx and Nedd4 for the ubiquitin-proteasome pathway, and IκBα, IκBβ, p50 and p65 subunits for the NF-κB pathway), as well as hypertrophy signalling (MyoD, myogenin and myostatin) were measured in the diaphragm of COPD patients in comparison to control subjects.

MATERIALS AND METHODS

Population

Diaphragm muscle biopsies were obtained from 19 patients with severe COPD and from 13 patients without COPD (controls). Biopsies were obtained during thoracotomy for lung transplantation in the COPD patients and during oesophagectomy or lobectomy for oesophageal and lung cancer, respectively (stage T1–2, N0–1 M0) in the patients without COPD. Informed consent was obtained from each participant. The study was approved by the Ethics Committee of the Universitaire Ziekenhuizen (Leuven, Belgium).

Pulmonary function testing was carried out prior to surgery. Patient characteristics are shown in table 1. 12 out of the 19 COPD patients were taking corticosteroids (methylprednisolone 4 mg·day^{-1} and 8 mg·day^{-1} for eight and four patients, respectively).

Diaphragm biopsies

Full-thickness biopsy specimens were obtained from the costal diaphragm. Part of the biopsy was frozen in isopentane for histological examination. The other section was frozen in liquid nitrogen and stored at -80°C to determine the role of markers of atrophy or hypertrophy signalling.

Histochemistry

Serial sections of the diaphragm were stained with haematoxylin and eosin to determine histological changes and with myofibrillar adenosine triphosphatase to measure fibre cross-sectional area and proportions.

RNA extraction and real-time quantitative PCR

Total RNA was isolated using the trizol method. Reverse transcription was performed with the Superscript III First-Strand Synthesis System (Invitrogen, Merelbeke, Belgium) according to the manufacturer's instructions.

Quantitative RT-PCR assay was performed on an ABI Prism 7700 Sequence Detection System, (Applied Biosystems, Lennik, Belgium) using the Platinum Sybr Green qPCR Supermix UDG kit (Invitrogen), according to the manufacturer's instructions. The primers used for this study are shown in table 2. Expression of the 18S gene was used to standardise the quantification of target cDNA.

Protein extraction

Approximately 40 mg of diaphragm was used for dual extractions of nuclear and cytoplasmic proteins with the NE-PER kit according to the manufacturers' instructions (Pierce Biotechnology, Erembodegem, Belgium). Protein concentration was determined using the Bradford method (Biorad, Nazareth, Belgium).

Gel electrophoresis and immunoblotting

Muscle proteins were separated on 12% SDS-polyacrylamide gels and transferred onto a polyvinylidene fluoride membrane.

TABLE 1 Anthropometric characteristics and pulmonary function tests in the controls and in the patients with chronic obstructive pulmonary disease (COPD)

Characteristics	Controls	COPD
Subjects n	13	19
Male/female n	11/2	8/11
Age yrs	64±3	58±1
Weight kg	71±3	58±2**
BMI kg·m^{-2}	25±1	21±1**
FEV_1 L	2.67±0.21	0.71±0.05$^{\#}$
FEV_1 % pred	98±5	26±2$^{\#}$
FEV_1/FVC %	76±2	33±2$^{\#}$
TLC % pred	95±6	126±5***
RV % pred	107±9	227±12¶
$D_{L,CO}$ % pred	83±5	29±2$^{\#}$
K_{CO} % pred	105±6	44±3$^{\#}$

Data are presented as mean±SEM, unless otherwise stated. BMI: body mass index; FEV_1: forced expiratory volume in 1 s; % pred: % predicted; FVC: forced vital capacity; TLC: total lung capacity; RV: residual volume; $D_{L,CO}$: diffusing capacity of the lung for carbon monoxide; K_{CO}: $D_{L,CO}$ per unit of alveolar volume. $^{\#}$: p<0.0001; ¶: p<0.00005; **: p<0.01; ***: p<0.001.

TABLE 2 Primer sequences used for real-time PCR

MAFbx	Forward: 5′ GTG GTA CTG AAA GTC CTT GAA GAC 3′
	Reverse: 5′ TTA ATG TTC CCG ACC AGC A 3′
MuRF1	Forward: 5′ GAA TAA CTG TAT CTC CAT GCT GG 3′
	Reverse: 5′ GGC ATA CAA CGT GTC AAA CTT 3′
MyoD	Forward: 5′ GAC GGC ATG ATG GAC TAC AG 3′
	Reverse: 5′ AGG CAG TCT AGG CTC GAC AC 3′
Myogenin	Forward: 5′ AGC GAA TGC AGC TCT CAC AG 3′
	Reverse: 5′ AGG TTG TGG GCA TCT GTA GG 3′
Nedd4	Forward: 5′ TTT GGA AAT TCA GCC GTG AG 3′
	Reverse: 5′ CCT GGT GGT AAT CCA GAT GAA 3′
Myostatin	Forward: 5′ TGT AAC CTT CCC AGG ACC AG 3′
	Reverse: 5′ GGT AAC GAC AGC ATC GTG ATT 3′

Membranes were incubated with the appropriate primary and secondary antibodies (see supplementary material) to detect either IκBα, IκBβ, MyoD, myogenin, myostatin or MAFbx. Proteins were visualised with the ECL Plus detection kit (GE Healthcare, Diegem, Belgium). Proper separation of nuclear and cytoplasmatic fractions was assessed while measuring Histone H3 (fig. E2 in supplementary material) and α-tubulin, respectively.

NF-κB activity

Activation of NF-κB p50 and p65 was detected using a microtitre plate assay by Pierce Biotechnology. Nuclear proteins were incubated in wells coated with double-stranded NF-κB consensus oligonucleotides. Bound p50 or p65 was identified with antibodies for the respective protein and detected with a horseradish peroxidase-coupled secondary antibody followed by ECL reagent, as recommended by the manufacturer. Luminescence signal was read in a luminometer (provided by F. Claessens; Labo Legendo, Leuven, Belgium).

Statistics

An unpaired t-test was used (data were normally distributed) to compare patients characteristics, and mRNA and protein expression between the two groups. Relationships were assessed with the Pearson correlation analysis. A p-value of $\leqslant 0.05$ was considered significant. Statistics were performed using the GraphPad software (version 4.01; GraphPad, San Diego, CA, USA).

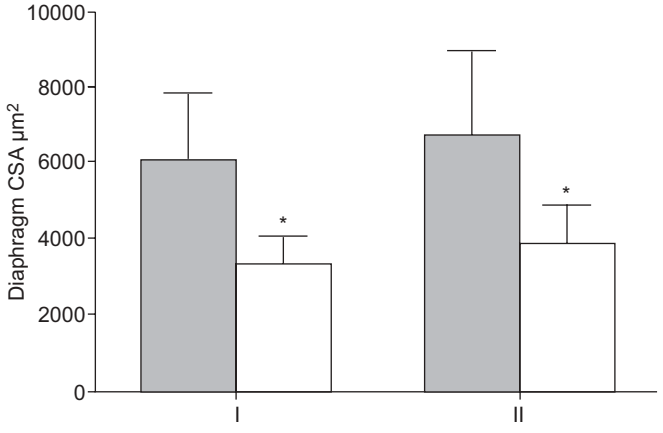

FIGURE 1. Cross-sectional area (CSA) of type I and type II fibres in the diaphragm of six controls (■) and six patients with severe chronic obstructive pulmonary disease (□). Data are presented as mean±SD. *: $p<0.05$.

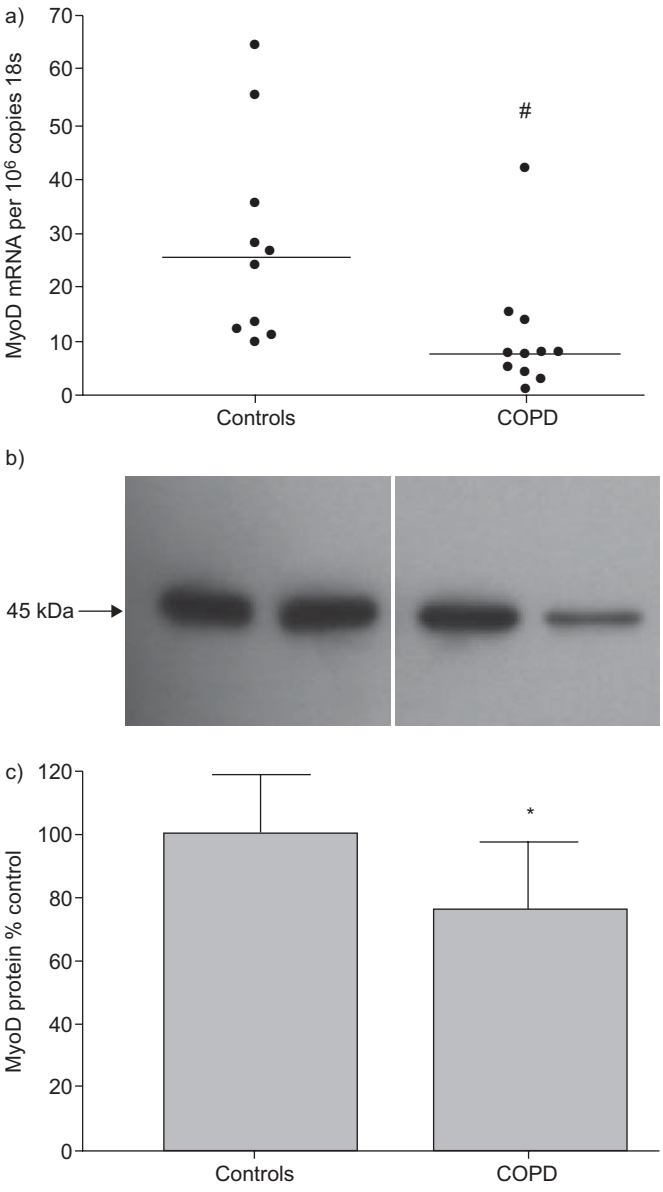

FIGURE 2. a) MyoD mRNA (expressed per 10^6 18s copies) and b, c) protein (relative to controls) expression in the diaphragm of controls and patients with chronic obstructive pulmonary disease (COPD). For mRNA, each data point represents an individual patient and horizontal lines represent the mean value. a) Controls: n=10; COPD: n=11. b, c) Controls: n=9; COPD: n=15. *: $p<0.05$; #: $p<0.005$.

RESULTS

Patient characteristics

Anthropometric characteristics and pulmonary function data are provided in table 1. The COPD patients did not differ from the controls with respect to height and age. Body mass index was significantly lower in the COPD patients. COPD patients

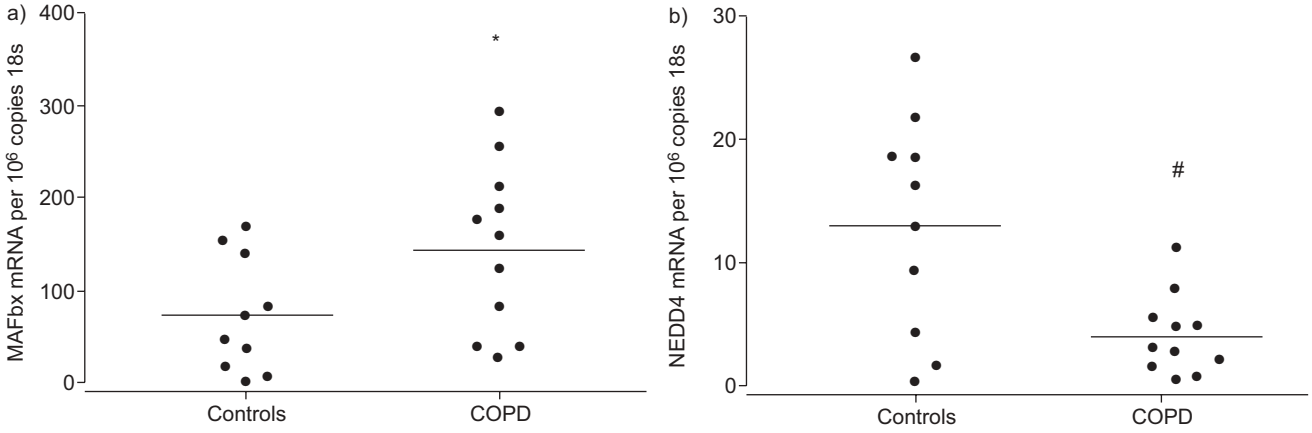

FIGURE 3. a) MAFbx mRNA expression and b) Nedd4 mRNA expression in the diaphragm of controls (n=10) and patients with chronic obstructive pulmonary disease (COPD; n=11). mRNA is expressed per 10^6 18s copies. Each data point represents an individual patient and the horizontal lines represent the mean value. *: $p<0.05$. #: $p<0.005$.

had severe (Global Initiative for Chronic Obstructive Lung Disease (GOLD) III) or very severe (GOLD IV) COPD.

Diaphragm histochemistry

As shown in figure 1, cross-sectional area of both type I and type II fibres was significantly reduced with 45% and 30%, respectively, in patients with COPD compared with controls. Moreover, there was a significantly higher proportion of type I fibres in the diaphragm of COPD patients in comparison with the control group (73% *versus* 48%) while the proportion of type II fibres was decreased.

MRFs

MyoD mRNA (-63%) (fig. 2a) and its protein content (-24%) (fig. 2b) were significantly decreased in the diaphragm of COPD patients, while there was no significant difference in myogenin mRNA and protein expression. An inverse relationship was found between diaphragmatic MyoD protein levels and diaphragm type I fibre proportion ($r= -0.65$, $p=0.04$).

Ubiquitin-proteasome pathway

MAFbx mRNA was significantly higher in the diaphragm of COPD patients (fig. 3a) while MuRF1 mRNA levels remained unchanged. MAFbx protein levels tended to be increased in the diaphragm of COPD patients (controls: $100\pm61\%$, COPD: $160\pm51\%$; $p=0.08$). Nedd4 mRNA was significantly decreased in the diaphragm of patients with COPD (fig. 3b).

NF-κB pathway

NF-κB p50 DNA-binding activity was decreased by 62% in the diaphragm of COPD patients (fig. 4a) while NF-κB p65 DNA-binding activity did not change. Cytoplasmatic levels of IκBα (-24%) (fig. 4b) and IκBβ (-58%) (fig. 4c) were decreased in the diaphragm of COPD patients.

Myostatin

In the diaphragm of COPD patients, myostatin mRNA was significantly decreased (-86%) in comparison with the control group (fig. 5a). Myostatin protein levels, both in the nuclear and cytoplasmatic fraction of the diaphragm, were significantly increased with 169% and 74%, respectively, in the COPD patients (fig. 5b and c).

DISCUSSION

The present study shows that atrophy and hypertrophy signalling is altered in the diaphragm of severe COPD patients. In particular, the NF-κB pathway, the ubiquitin-proteasome pathway and myostatin were activated while MyoD signalling was depressed. Diaphragm atrophy and fibre adaptation towards a slower profile was also observed.

Diaphragm fibre shift and atrophy

In agreement with previous studies [5], the proportion of type I fibres was higher in the diaphragm of the COPD patients in this study. This adaptation has been regarded as beneficial because the diaphragm of COPD would be more resistant to fatigue [1]. This shift towards a slower profile has also been observed at the level of the SERCA pumps [3]. It has also been shown that resting energy expenditure, as measured by diaphragm tension-time index, and the neural drive to the diaphragm are increased in severe COPD at rest [4, 10]. These adaptations have been suggested to result from continuous overload of the diaphragm due to COPD. The diaphragm of severe COPD patients appears to undergo long-term moderate continuous endurance training [5]. In limb muscles, endurance training results in a shift from fast to slow fibre type [11]. A major difference between the diaphragm of COPD patients and limb muscle studies is the long-term and continuous training undergone by the diaphragm and the age of the population, as severe COPD affects elderly individuals. In addition, in the lifelong trained elderly, endurance training results in a greater proportion of type I fibre in vastus lateralis muscle [11].

In the present study, cross-sectional area of type I and type II fibres was decreased in the diaphragm of the COPD patients as previously described [5]. These data indicate that despite continuous overload resulting in increased diaphragm activity, diaphragm atrophy develops in COPD patients.

The MRFs MyoD and myogenin

The data of the present study suggest that MyoD is probably implicated in the fibre shift seen in the diaphragm of COPD

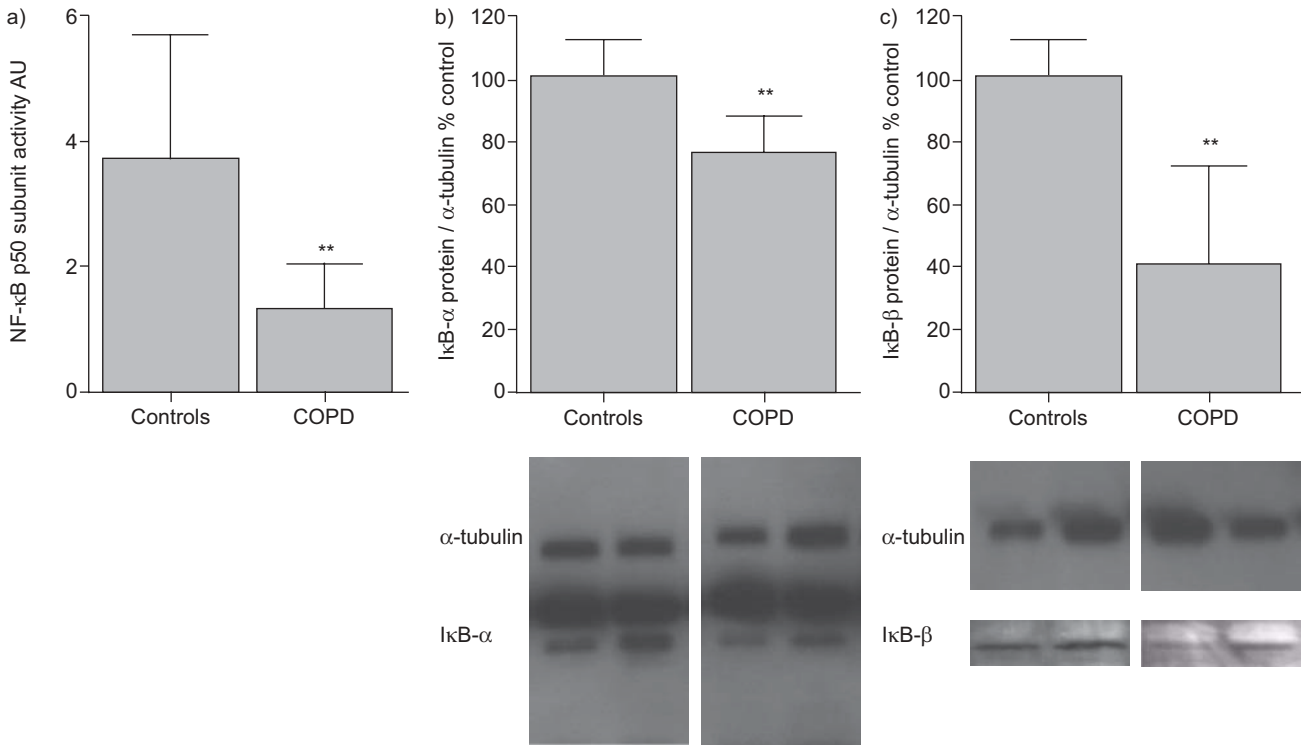

FIGURE 4. a) Nuclear factor (NF)-κB p50 DNA-binding activity and protein expression of inhibitor proteins b) IκBα (35 kDa) and c) IκBβ (45 kDa) (normalised to α-tubulin (55 kDa) and expressed relative to controls) in the diaphragm of controls (n=9) and patients with chronic obstructive pulmonary disease (COPD; n=15). Data are presented as mean±SD. **: $p<0.01$.

patients. In skeletal muscle, the alterations in MyoD and myogenin are suggested to be a drive for fibre shifting under conditions such as denervation and unloading [8]. In this regard, a decrease in MyoD will lead to a shift towards a slower, more oxidative profile [8]. Our study shows decreased MyoD mRNA and protein levels in the diaphragm of the COPD patients which is compatible with the increased proportion of type I fibres. The inverse relationship between MyoD protein levels and the type I proportion found in the present study further supports a role for MyoD in fibre shifting. A decrease in diaphragmatic MyoD protein levels has been recently shown in a hamster model of emphysema [12].

In addition, the calcineurin-nuclear factor activate T-cells (NFAT) pathway, known to specifically induce the slow-twitch muscle programme, may have played a role in the diaphragm fibre shift observed in the COPD patients. Overload of the diaphragm in these patients may have activated calcineurin while increasing intracellular calcium. Indeed, it is well-known that calcineurin acts as a sensor of sustained elevated intracellular calcium concentration. Although not investigated in the present study, the role of the calcineurin-NFAT should not be neglected.

Myostatin

In the present study, myostatin mRNA levels in the diaphragm of COPD patients were decreased while its protein expression was increased. Differences in myostatin mRNA and protein expression levels have been previously reported in skeletal muscle atrophy [13, 14]. Discrepancy is probably related to the auto-regulation mechanism of myostatin based on a negative feedback loop with increasing levels of active myostatin protein resulting in downregulation of myostatin mRNA expression [15]. The data from this study are in agreement with these findings.

The lower myostatin mRNA levels found in the diaphragm of the COPD patients is compatible with the increased proportion of type I fibres as myostatin is mainly expressed in fast-type fibres [16]. Myostatin could play a role in modulating gene expression controlling muscle fibre type. Moreover, the myostatin gene has been reported to be a target gene of MyoD as expression of myostatin can be activated by MyoD [17]. The lower levels of diaphragmatic MyoD in our COPD patients could contribute to the downregulation of myostatin mRNA.

Surprisingly, mRNA levels of myostatin, known as a negative regulator of muscle growth, were decreased in the diaphragm of COPD patients despite the presence of atrophy. In fact, previous studies on peripheral muscles are consistent with our observation [18, 19]. It has even been suggested that the role of myostatin would rather be the inhibition of hypertrophy than the induction of atrophy, since increased myostatin concentration was not sufficient for muscle mass loss.

However, in the present study, myostatin protein levels in the nuclear and cytoplasmic fraction were up-regulated in the diaphragm of the COPD patients, and may possibly have contributed to the observed diaphragm atrophy. Indeed, several studies have reported enhanced myostatin levels in

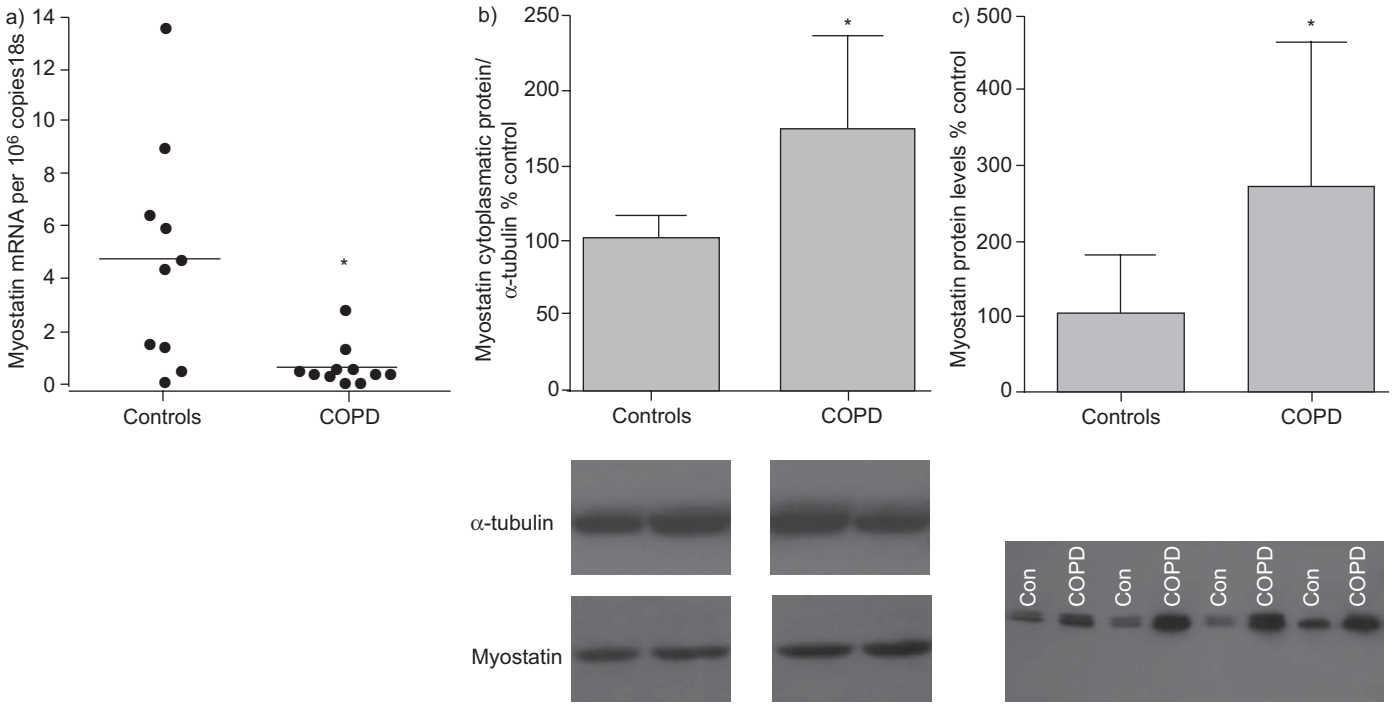

FIGURE 5. a) Myostatin mRNA (expressed per 10^6 18S copies) and myostatin protein levels (26 kDa) in b) cytoplasmic and c) nuclear fraction in the diaphragm of controls and patients with chronic obstructive pulmonary disease (COPD). Each data point in a) represents an individual patient. Controls: n=10; COPD: n=11. Horizontal lines represent the mean value. a, c) Controls: n=9; COPD: n=15. Data normalised to α-tubulin (c; 55 kDa) expressed relative to controls presented as mean±SD. Con: control. *: $p<0.05$.

animals after disuse [9] and also in patients with chronic muscle atrophy [9].

Finally, the presence of myostatin protein in the nuclear fraction is intriguing but it is not new [13, 16]. Indeed, myostatin protein was found to be essentially located in the nucleus of the myotubes [16], while in rat muscle it was clearly more expressed in the nuclear pellet than in the cytoplasmic fraction or the total homogenate [13]. Further experiments are needed to determine the implication of nuclear myostatin.

NF-κB pathway

In the present study, decreased content of the cytoplasmatic IκBα and IκBβ was observed in the diaphragm of the COPD patients, suggesting enhanced NF-κB signalling. IκBα content was less decreased than IκBβ. In fact, IκBα has a κB recognition sequence in its promoter region and thus, resynthesis of IκBα can be induced by NF-κB; however, this is not the case for IκBβ [20]. NF-κB p50 DNA-binding activity was reduced in the diaphragm of COPD patients in the present study, while p65 DNA-binding activity remained unchanged. However, the p50 subunit is able to dimerise with another p50 subunit, and these homodimers inhibit gene transcription [21]. Similar findings were reported in the vastus lateralis of patients with diabetes type 2 and low-grade chronic inflammation [22]. Interestingly, suppression of MyoD mRNA *via* NF-κB has been described [23]. Thus, the increased NF-κB activity could contribute to the decreased diaphragmatic MyoD protein levels in the COPD patients. Activation of NF-κB also decreased MyoD protein stability and so could promote MyoD degradation [24]. Increased NF-κB activity has been reported in the quadriceps of COPD patients with low body weight, suggesting a role for NF-κB in skeletal muscle atrophy in these patients [25].

The ubiquitin-proteasome pathway

Upregulation of MAFbx mRNA was found in the diaphragm of COPD patients while MuRF1 mRNA was unchanged. These data are in line with the findings previously reported in mild-to-moderate COPD patients and are also compatible with the increased protein ubiquitination reported in the diaphragm of COPD patients [5]. Interestingly, MAFbx has been associated with specific degradation of myosin [26]. Therefore, upregulation of MAFbx in the diaphragm of COPD patients is in line with the reduction of diaphragm myosin content reported in these patients [27]. Conversely, MuRF1 has been found to target specific subsets of myofibrillar proteins, such as titin [28]. Preserved diaphragm titin content in COPD patients [27] is in agreement with unaltered MuRF1 mRNA expression.

In the present study, Nedd4 mRNA was significantly down-regulated in the diaphragm of the COPD patients. Nedd4 is a homologous to E6-AP carboxyl terminus domain containing E3 ubiquitin-protein ligase which has been shown to be up-regulated in atrophy caused by reduced muscle tension, but not in cachexia atrophy [6]. Decreased passive diaphragm tension is present in COPD patients [27]. Reduced muscle tension may possibly involve integrin signalling or titin mechanotransduction. In COPD patients, alternative splicing of the titin gene leading to a modulation of the titin's spring segment has been proposed as a potential contributor to the decreased passive tension seen in stretched diaphragm fibre [27]. Thus, it seems that this reduction in titin stretching does

not induce an up-regulation of Nedd4 in the present study. These data suggest that the decrease in passive force generation reported in the diaphragm of COPD patients will not induce activation of the ubiquitin-proteasome pathway by upregulation of Nedd4.

Potential triggers

There are several factors related to COPD that might have affected the different pathways investigated in the present study. First, hypoxia, a common feature in COPD patients, is known to inhibit the expression of muscle regulatory factors [29] and to activate the NF-κB pathway [30]. Secondly, diaphragm shortening caused by pulmonary hyperinflation may have affected some of these pathways. Indeed, in chronic hyperinflation, the resting sarcomere length in human diaphragm muscle fibres decreases according to the degree of pulmonary hyperinflation [31]. Increased proteolysis may be involved in sarcomere deletion. As such, diaphragm shortening may have indirectly contributed to the activation of the ubiquitin-proteasome pathway. In addition, increased mechanical load caused by pulmonary hyperinflation may have also contributed to changes in MyoD and/or myostatin expression while mimicking the effect of endurance training. There are, however, no clear data in the literature supporting this concept. In addition, corticosteroid known to up-regulate MAFbx and MuRF1 [32], and to selectively decrease MyoD expression [33] and up-regulate myostatin gene expression [9] may have contributed to the alterations reported in our study. Obviously, chronic inflammation, as occurs in COPD patients, may have altered several of these pathways as well. In particular, circulating cytokines are known to stimulate proteolysis through the ubiquitin-proteasome pathway [34] and to destabilise MyoD [24].

In conclusion, this study showed that diaphragm fibre atrophy and fibre adaptation towards a slower profile are present in the diaphragm of severe COPD patients. In addition, several signalling pathways are altered in the diaphragm of COPD patients. These include up-regulation of the NF-κB pathway, the ubiquitin-proteasome pathway and myostatin, and down-regulation of MyoD. Taken together, these alterations may contribute to diaphragm dysfunction in COPD patients.

SUPPORT STATEMENT

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STATEMENT OF INTEREST

A statement of interest for M. Decramer can be found at www.erj.ersjorurnals.com/misc/statements.dtl

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