

**ACTIVATION OF UBIQUITIN-PROTEASOME PATHWAY IN THE DIAPHRAGM
IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE**

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ABSTRACT (Word count: 248)

RATIONALE Recent studies show that myosin content of the diaphragm in patients with mild to moderate COPD is reduced, compromising diaphragm contractile performance. The mechanisms for reduced contractile protein content are unknown. In the present study we hypothesized that the loss of contractile protein content is associated with activation of the ubiquitin-proteasome pathway in the diaphragm of patients with mild-to-moderate COPD.

METHODS Proteolytic activity of isolated 20S proteasomes was determined in diaphragm biopsies from patients with and without COPD (predicted mean FEV₁ 66% and 93%, respectively). In addition, we determined 20S proteasome subunit C8 protein levels by means of Western blotting, ubiquitin-ligase mRNA levels by means of real-time PCR, and caspase-3 activity by determining the hydrolysis of fluorogenic substrates.

RESULTS 20S proteasome activity was ~3-fold increased in the diaphragm of patients with COPD. C8 protein levels were not significantly different between COPD and non-COPD diaphragm, indicating increased specific activity of individual proteasomes, rather than an increased number of proteasomes. mRNA levels of the muscle-specific ubiquitin-ligase MAFbx were significantly higher in diaphragm from COPD patients compared non-COPD patients. Caspase-3-mediated cleavage of actomyosin complexes is considered an initial step in muscle wasting, yielding fragments which can be degraded by the ubiquitin-proteasome pathway. In line with the increased ubiquitin-proteasome activity, caspase-3 activity was higher in diaphragm homogenates from patients with COPD. **CONCLUSIONS** The present study is the first to demonstrate increased activity of the ubiquitin-proteasome pathway in COPD diaphragm. Importantly, these changes occur in patients with only mild-to-moderate COPD (GOLD I/II).

Key words: COPD, proteolysis, caspase-3, diaphragm function, myosin

INTRODUCTION

Diaphragm weakness is associated with dyspnea, increased morbidity and -mortality in patients with severe chronic obstructive pulmonary disease (COPD) ^{1;2}. Traditionally, diaphragm weakness has been ascribed to hyperinflation-induced diaphragm shortening, which places the diaphragm on a suboptimal position on its force-length curve ^{3;4}. Recent studies show that structural, biochemical and functional alterations may contribute to impaired diaphragm function in COPD patients ⁵⁻¹².

Force generating capacity is strongly dependent on contractile protein content of the muscle fibers ¹³. Recent work from our lab demonstrated that diaphragm fibers from patients with mild-to-moderate COPD (GOLD I/II) have ~30% reduced myosin content ^{8;9}, which compromises diaphragm single fiber force generation ⁹. This diaphragm wasting indicates an imbalance between myosin synthesis and degradation in COPD.

In animal models of muscle atrophy selective down regulation of myosin has been found concurrent with accelerated protein degradation by the ubiquitin proteasome pathway ¹⁴⁻¹⁸. Proteins degraded by the ubiquitin-proteasome pathway are first linked to a chain of ubiquitin molecules, which marks them for subsequent degradation by the 26S proteasome. Ubiquitination of proteins is regulated by multiple enzymes. Among these enzymes, the ubiquitin-ligases (E3-ligases) are particularly important because they account for substrate specificity of protein degradation ¹⁹. In recent animal studies, the expression of two newly discovered muscle-specific E3-ligases, MAFbx (atrogen-1) and MURF-1, was substantially increased in peripheral skeletal muscle during various muscle wasting conditions ^{17;20-22}. Therefore, together with increased proteasome activity, elevated MAFbx and MURF-1 mRNA levels are reliable molecular markers for muscle atrophy ^{16;23}.

Previously, we demonstrated increased protein ubiquitination in COPD diaphragm ⁹. This might suggest activation of the ubiquitin-proteasome pathway, but it could also be indicative

of reduced proteasome activity leading to accumulation of ubiquitinated proteins. To establish, for the first time, the activity level of the ubiquitin-proteasome pathway in COPD diaphragm, we investigated several of its key components in the diaphragm of patients with COPD. We hypothesized that proteasome activity is increased, and that E3-ligase mRNA levels are elevated in the diaphragm of patients with mild-to-moderate COPD.

The sequence of events leading to muscle wasting involves additional mechanisms to be at play, because the ubiquitin-proteasome pathway does not break down intact sarcomeres ²⁴. It has been postulated that disassociation of myosin and actin filaments from the muscle sarcomere is the rate-limiting step in muscle protein degradation. Recent evidence indicates that activation of caspase-3 and results in cleavage of these myofilaments and yields fragments that are degraded by the ubiquitin-proteasome pathway ²⁵. Therefore, we hypothesized that in addition to activation of the ubiquitin-proteasome pathway, caspase-3 activity is increased in COPD diaphragm.

METHODS (Word count: 331)

Subjects, pulmonary function testing, and diaphragm biopsies

Diaphragm muscle biopsies were obtained from 15 patients with COPD and 13 patients without COPD. Biopsies were obtained from the right anterior costal diaphragm during thoracotomy for lung cancer (stage T₁₋₂N₀₋₁M₀ in both groups). The fresh biopsy was rapidly frozen in liquid nitrogen-cooled isopentane and stored at -80°C for later analysis. Exclusion criteria included weight loss of more than 10% in the last six months before surgery, neuromuscular diseases, thyroid diseases and chronic heart failure. None of the subjects received chemotherapy prior to 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.

Isolation of 20S proteasomes and measurement of proteolytic activity

The 20S proteasome isolation and proteolytic activity measurements were determined according to Hobler et al.²⁶, with minor modifications. In short, the proteolytic activity of isolated 20S proteasomes was determined by measuring the activity against the fluorogenic substrates succinyl-leu-leu-val-tyr-7-amido-4-methylcoumarin (LLVY) and N-carbenzoxyleu-leu-glu-7-amido-4-methylcoumarin (LLE) (Sigma-Aldrich, Zwijndrecht, the Netherlands). For additional details see online supplement.

MAFbx and MURF-1 mRNA determination with real-time quantitative PCR

The methodology for MAFbx and MURF-1 mRNA determinations are presented in the online supplement.

Western blot analysis

Myosin heavy chain and 20S proteasome subunit C8 content were determined semiquantitatively by Western blotting as presented in the online supplement.

Measurement of caspase-3 activity

Caspase-3 activity was determined as described by Du et al.²⁵, with minor modifications. In short, cleavage of the fluorogenic substrate N-acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) by diaphragm homogenates was determined with a spectrophotometer. See online supplement for details.

Statistical methods

To evaluate statistical significance of difference between patients with and without COPD a t-test was used (data were normally distributed). Because of limited diaphragm tissue available per patient, data from proteasome activity, caspase-3 activity, E3-ligase, and Western blotting studies are not based on the same patients, although there is extensive overlap. $P < 0.05$ was used as criterion for statistical significance.

RESULTS

Subject characteristics

Patient characteristics and pulmonary function data are shown in Table 1. COPD patients included for proteasome activity, E3-ligase, caspase-3 activity and western blotting studies were classified as mild (stage I) or moderate COPD (stage II) on the basis of the GOLD classification²⁷.

Myosin heavy chain protein levels

In line with previous data from our lab^{8;9}, myosin heavy chain content was significantly lower in the diaphragm of COPD patients compared to non-COPD patients (figure 1).

20S proteasome activity

The proteasome activity against the substrate LLVY was ~2-fold higher, and that against LLE was ~3-fold higher in COPD diaphragm compared to non-COPD diaphragm ($p < 0.05$, figure 2). These data indicate that the chymotrypsin-like (LLVY) and peptidylglutamyl-peptide hydrolyzing (LLE) peptidase activities of the proteasome are increased in the diaphragm of patients with COPD. For representative proteasome activity vs time plots, see figure E1 in online supplement.

Proteasome subunit C8 protein levels

To investigate if changes in proteasome activity are the result of changes in the number of proteasomes, we determined the protein level of the C8 subunit of the 20S proteasome. Figure 3 demonstrates that the protein levels of the 20S proteasome C8 subunit were not significantly different between COPD and non-COPD diaphragm. These data suggest that the increased

proteasome activity is the result of increased *specific activity* of the individual proteasomes, rather than of an increased *number* of proteasomes.

MAFbx and MURF-1 mRNA

We determined MAFbx and MURF-1 mRNA levels by means of real-time quantitative PCR. Compared with the non-COPD patients, patients with COPD had higher levels of MAFbx mRNA ($p < 0.05$, figure 4). MURF-1 mRNA levels were not significantly different between COPD and non-COPD patients (figure 4).

Caspase-3 activity

Caspase-3 activity was assessed in diaphragm homogenates by determining the cleavage rate of the fluorogenic substrate Ac-DEVD-AMC. Caspase-3 activity of diaphragm homogenates was ~1.5-fold higher for COPD patients compared to non-COPD patients ($p < 0.05$, figure 5). For representative caspase-3 activity vs time plots, see figure E2 in online supplement.

DISCUSSION

This study is the first to reveal several key findings with regard to diaphragm muscle proteolysis in humans, particularly in patients with COPD. We found that reduced myosin content was associated with ubiquitin-proteasome pathway activation in the diaphragm in COPD, as indicated by increased MAFbx mRNA expression and proteasome activity.

Recent evidence indicates that activation of caspase-3 is an initial step in myofilament proteolysis by cleavage of myosin and actin ²⁵. In this way, activated caspase-3 yields fragments that are degradable by the ubiquitin-proteasome pathway. Indeed, we found elevated caspase-3 activity in the diaphragm of patients with COPD compared to non-COPD patients. Importantly, activation of ubiquitin-proteasome pathway and caspase-3 activity occurred in patients with only mild-to-moderate COPD (GOLD stage I/II).

Increased activity of the ubiquitin-proteasome pathway in COPD diaphragm

In line with previous work from our lab ^{8;9}, the present study confirms reduced myosin content of the diaphragm in patients with COPD. As a result, diaphragm fibers from these patients have impaired force generating capacity⁹, contributing to diaphragm weakness.

In many conditions associated with muscle wasting, such as sepsis, cancer, and chronic heart failure, loss of contractile protein occurs largely through the ubiquitin-proteasome pathway ²⁸.

This ATP-dependent proteolytic pathway can be divided in two major steps. In the first step, proteins are marked for degradation by conjugation of multiple ubiquitin molecules. This ubiquitin-protein conjugation is achieved by the combined action of ubiquitin-activating enzymes (E1), the ubiquitin-conjugating enzymes (E2), and ubiquitin-ligases (E3). In the second step, ubiquitinated proteins are recognized and subsequently degraded by the 26S proteasome, a large multi-catalytic protein complex consisting of 2 regulatory 19S complexes

and a 20S proteolytic core ²⁹. Animal studies have shown that muscle wasting-associated conditions are characterized by a significant upregulation of several key components of the ubiquitin-proteasome pathway ^{16;24;26;30;31}. The present data show increased proteasome activity in the diaphragm of patients with only mild-to-moderate COPD. *In vitro* 20S proteasome activities measured under similar conditions as reported here have been in good agreement with rates of proteolysis ^{32;33}.

The observed upregulation of the E3-ligase MAFbx in COPD diaphragm strongly supports our notion that the ubiquitin-proteasome pathway is activated in these patients. Of all the proteins involved in ubiquitinating protein substrates, E3-ligases seem to have the greatest tissue and substrate specificity ¹⁹. MAFbx has been found to be markedly upregulated during cachexia- and disuse-induced atrophy ^{17;20}. Moreover, when MAFbx knockout mice were subjected to an atrophic stimulus, muscle atrophy was attenuated by 50-60%, indicating an important role for this E3-ligase in atrophy ²⁰. Previous studies in animals have shown parallel upregulation of MAFbx and another muscle-specific E3-ligase, MURF-1, during multiple types of atrophy ^{16;20}. In contrast to MAFbx, diaphragmatic MURF-1 mRNA levels were not different between COPD and non-COPD patients. This differential response of MAFbx and MURF-1 in atrophying COPD diaphragm is in line with recent work on E3-ligase expression in skeletal muscle atrophy in humans ^{34;35}. Together, these data suggest that human skeletal muscle atrophy is regulated more by MAFbx than by MURF-1. Interestingly, MURF-1 has been suggested to target a specific set of myofibrillar proteins, including titin but not myosin ³⁶, whereas MAFbx has been associated with specific degradation of myosin ³⁷. Previous data from our group indicating preserved titin but reduced myosin content in COPD diaphragm ⁸ are in line with this concept.

Increased caspase-3 activity in COPD diaphragm

Although the bulk of contractile protein degradation in several models of atrophy involves the ubiquitin-proteasome pathway, this proteolytic pathway can not degrade intact myosin or actin²⁴. Recent evidence suggests that caspase-3²⁵ and calpains³⁸ play a role in contractile protein degradation upstream of the ubiquitin-proteasome pathway (for a schematic representation of the proposed roles of these proteolytic systems in muscle atrophy, see Jackman & Kandarian³⁹). Du et al.²⁵ have shown that caspase-3 activation is an initial step in contractile protein degradation by cleavage of actomyosin complexes, thereby rendering actin fragments. Degraded actomyosin complexes can be ubiquitinated and degraded in the 26S proteasome complex. As a result, caspase-3-mediated contractile protein cleavage has been proposed to be an early rate-limiting step in contractile protein degradation during muscle atrophy. Our data provide the first evidence of caspase-3 activation in the diaphragm of patients with COPD. The co-activation of caspase-3 and the ubiquitin-proteasome pathway in COPD diaphragm is in line with previous studies investigating mechanisms of muscle atrophy^{25;40}, and supports a role for caspase-3 in the loss of diaphragmatic contractile protein content in these patients.

Potential triggers for activation of the ubiquitin-proteasome pathway and caspase-3 in COPD diaphragm

Potential triggers for the observed activation of the ubiquitin-proteasome pathway and caspase-3 in COPD diaphragm might include increased local generation of pro-inflammatory cytokines and reactive oxygen species. Recent studies show increased mRNA levels of tumor necrosis factor- α and its receptors in the diaphragm of patients with moderate COPD⁴¹. Interestingly, the selective loss of myosin during cancer cachexia appears to be mediated by cytokine-induced activation of the ubiquitin-proteasome pathway, such as induction of

MAFbx¹⁴. It could be speculated that increased local expression of pro-inflammatory cytokines triggers the ubiquitin-proteasome pathway and the loss of myosin in the diaphragm of patients with mild-to-moderate COPD. Also, oxidative stress has been shown to occur in the diaphragm of patients with severe COPD¹⁰. Like pro-inflammatory cytokines, reactive oxygen species are known stimulators of the ubiquitin-proteasome pathway in muscle^{42;43} and they might therefore contribute to diaphragm wasting in these patients.

Therapeutic implications: a role for inhibitors of the ubiquitin-proteasome pathway or caspase-3 activity ?

Recently, the proteasome inhibitor bortezomib, has been approved for treatment of multiple myeloma in humans⁴⁴. Animal studies have shown that *in vivo* administration of bortezomib in a model of disuse-atrophy prevented muscle wasting by ~50%⁴⁵. Similar results were found with administration of bortezomib in a rat model of denervation-atrophy⁴⁶. In both studies bortezomib was well tolerated and no signs of toxicity were observed. These findings show promise for the use of proteasome inhibitors in syndromes associated with muscle wasting, such as the diaphragm in COPD.

Theoretically, inhibition of E3-ligases rather than the proteasome provides an ideal drug target, because E3-ligases have very high substrate selectivity. Therefore, a specific inhibitor of for instance MAFbx should be a highly specific drug with probably few side effects, and might prove beneficial in preserving contractile protein content and preventing diaphragm weakness in COPD diaphragm. Unfortunately, to date no ubiquitin-ligase inhibitor has reached the clinic.

Finally, Supinski and Callahan⁴⁷ have recently shown that *in vivo* inhibition of caspase-3 activity attenuated sepsis-induced diaphragm weakness. This new finding might provide a therapeutic target upstream of the ubiquitin proteasome pathway.

In conclusion, the present study for the first time demonstrates increased activation of the ubiquitin-proteasome pathway and increased caspase-3 activity in the diaphragm of patients with mild-to-moderate COPD. These findings provide a potential molecular mechanism underlying the development of diaphragm fiber atrophy and weakness in these patients.

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LEGENDS TO FIGURES

Figure 1. Myosin heavy chain content in COPD and non-COPD diaphragm. Myosin heavy chain content was determined in diaphragm homogenates by Western blotting and subsequent densitometric quantification of protein bands. *Upper panel:* Myosin heavy chain content in diaphragm homogenates from COPD patients vs. non-COPD patients. *Lower panel:* representative myosin heavy chain immunoblot from diaphragm homogenates from a COPD and non-COPD patient. For the positive control purified myosin heavy chain was used. For the negative control a homogenate from a non-COPD patient was stained without addition of the primary (anti-myosin heavy chain) antibody. Data are presented as mean \pm sem. *: $P < 0.05$ difference from non-COPD.

Figure 2. Proteasome activity in the diaphragm of COPD and non-COPD patients. Proteasome activity against fluorogenic substrates succinyl-leu-leu-val-tyr-7-amido-4-methylcoumarin (LLVY) and *N*-carbenzoxymethyl-leu-leu-glu-7-amido-4-methylcoumarin (LLE) was higher in COPD (black bars) vs. non-COPD (white bars) patients. AMC = amido-4-methylcoumarin. Data are presented as mean \pm sem. *: $P < 0.05$ difference from non-COPD.

Figure 3. 20S proteasome subunit C8 protein content in the diaphragm of COPD and non-COPD patients. Subunit C8 protein content was determined in diaphragm homogenates by Western blotting and subsequent densitometric quantification of protein bands. *Upper panel:* our analyses revealed comparable contents of subunit C8 in the diaphragm of COPD (black bar) and non-COPD (white bar) patients. *Lower panel:* representative C8 subunit immunoblot from diaphragm homogenates from a COPD and non-COPD patient. For the positive control we used an isolated proteasome fraction from a non-COPD patient (the same fraction as was

used for the proteasome-activity assays, see figure 2). For the negative control a homogenate from a non-COPD patient was stained without addition of the primary (anti-C8) antibody. Data are presented as mean \pm sem. *: $P < 0.05$ difference from non-COPD.

Figure 4. E3-ligase mRNA levels in the diaphragm of COPD and non-COPD patients. MAFbx mRNA levels are higher in COPD (black bars) vs. non-COPD (white bars) diaphragm. MURF-1 mRNA levels were not significantly different between COPD and non-COPD diaphragm. Data are presented as mean \pm sem. *: $P < 0.05$ difference from non-COPD.

Figure 5. Caspase-3 activity in the diaphragm of COPD and non-COPD patients. Caspase-3 activity against the fluorogenic substrate N-acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) was higher in the diaphragm homogenates from COPD patients (black bars) vs. non-COPD patients (white bars). Data are presented as mean \pm sem. *: $P < 0.05$ difference from non-COPD.

TABLE

Table 1. Patient Characteristics.

	Proteasome activity		E3-ligase		Caspase-3 activity		Western blotting	
	Non-COPD (n = 7)	COPD (n = 7)	Non-COPD (n = 10)	COPD (n = 11)	Non-COPD (n = 7)	COPD (n = 7)	Non-COPD (n = 9)	COPD (n = 9)
Male/female	6 / 1	6 / 1	9 / 1	9 / 2	6 / 1	7 / 0	8 / 1	9 / 0
Age, yr	64 ± 3	66 ± 2	63 ± 3	65 ± 3	66 ± 3	68 ± 2	65 ± 3	66 ± 2
BMI, kg*m ⁻²	28 ± 1	28 ± 2	28 ± 1	26 ± 2	28 ± 1	28 ± 2	28 ± 1	27 ± 2
FEV ₁ , % predicted	93 ± 4	66 ± 7	98 ± 4	63 ± 4	97 ± 4	70 ± 7	96 ± 4	67 ± 6
VC, % predicted	91 ± 4	93 ± 4	88 ± 4	83 ± 6	95 ± 5	96 ± 6	91 ± 4	95 ± 5
FEV ₁ /VC, %	77 ± 2	56 ± 3	76 ± 2	54 ± 2	74 ± 1	55 ± 3	76 ± 2	54 ± 3
TLC, % predicted	86 ± 3	111 ± 4	91 ± 4	106 ± 4	93 ± 5	99 ± 4	91 ± 4	107 ± 5
RV/TLC, %	33 ± 3	47 ± 2	38 ± 2	47 ± 3	36 ± 3	45 ± 4	37 ± 2	44 ± 3
D _{Lco} /VA, % predicted	103 ± 9	78 ± 8	112 ± 5	76 ± 7	92 ± 5	84 ± 10	103 ± 6	79 ± 8
Pao ₂ , kPa	11.2 ± 0.3	10.2 ± 0.5	10.2 ± 0.5	10.5 ± 0.5	10.3 ± 0.6	10.6 ± 0.6	10.2 ± 0.5	10.4 ± 0.3
Paco ₂ , kPa	4.8 ± 0.1	5.1 ± 0.1	4.7 ± 0.1	5.0 ± 0.2	4.6 ± 0.1	4.8 ± 0.2	4.7 ± 0.1	5.2 ± 0.2

Patient characteristics for proteasome activity, E3-ligases, caspase-3 activity, and Western blotting studies. Values are means ± SEM. Definition of abbreviations: BMI = body mass index; FEV₁ = forced expiratory volume in first s; VC = vital capacity; TLC = total lung capacity; RV = residual volume; D_{Lco}/VA = carbon monoxide transfer coefficient per alveolar volume; Pao₂ = arterial Po₂; Paco₂ = arterial Pco₂.

FIGURES

Figure 1

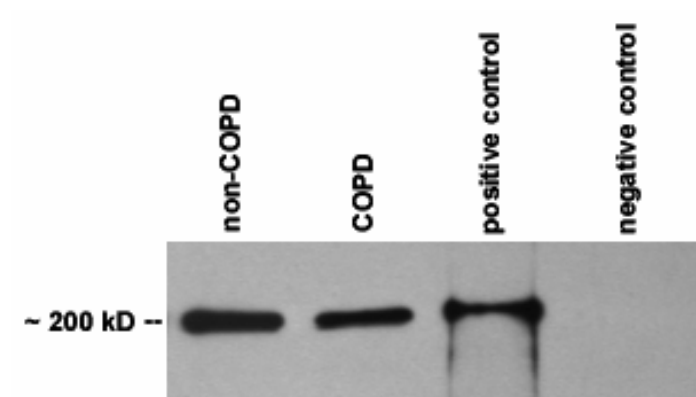
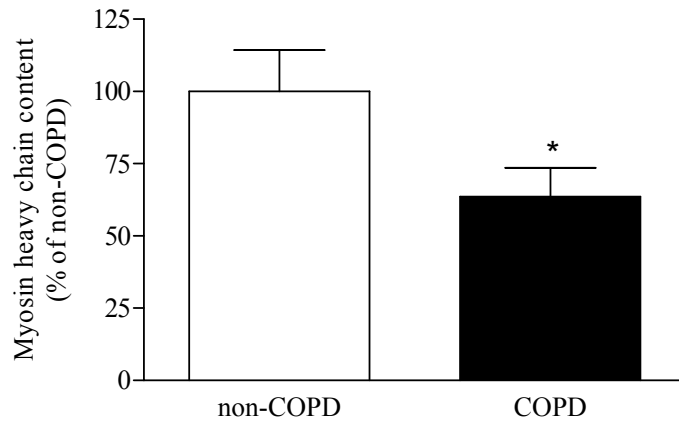


Figure 2

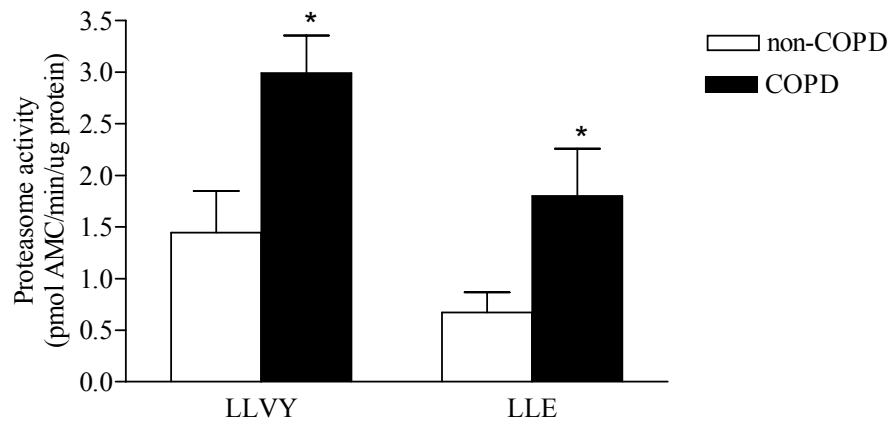


Figure 3

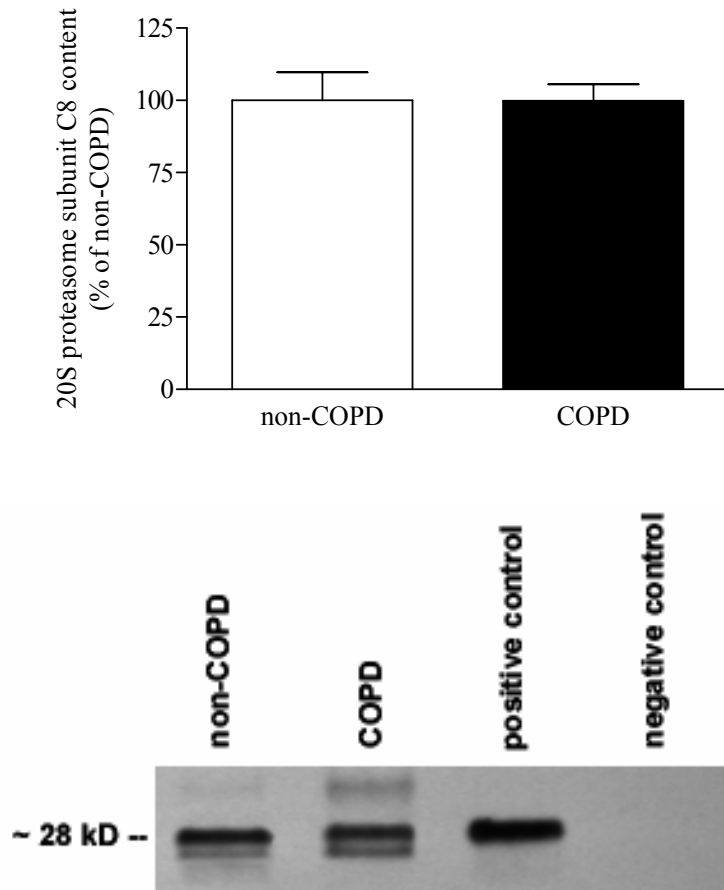


Figure 4

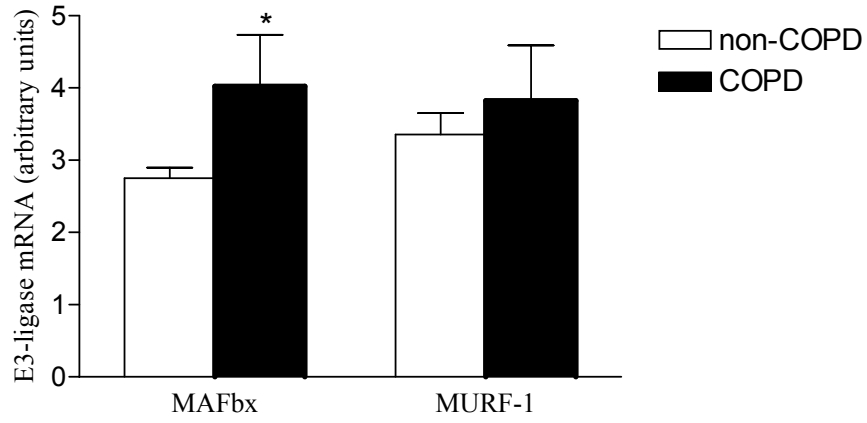
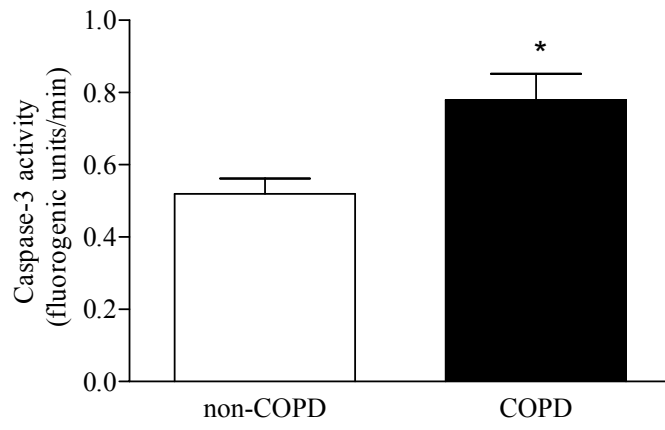


Figure 5



**ACTIVATION OF UBIQUITIN-PROTEASOME PATHWAY IN THE DIAPHRAGM
IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE**

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Online data supplement

METHODS

Isolation of 20S proteasomes and measurement of proteolytic activity

The 20S proteasome isolation and proteolytic activity measurements were determined according to Hobler et al. ¹, with minor modifications. To isolate 20S proteasomes, diaphragm samples were homogenized in ice-cold buffer (pH 7.5) containing (in mM) 50 Tris-HCl, 5 MgCl₂, 250 sucrose, 1 dithiothreitol, 0.2 phenylmethylsulphonylfluoride, and protease inhibitor cocktail (Sigma-Aldrich, Zwijndrecht, the Netherlands) by means of a Dounce homogenizer. Subsequently, the proteasomes were isolated from the homogenates by three sequential centrifugation steps; the first centrifugation was at 10,000 g for 20 min. The supernatant was centrifuged at 100,000 g for 1h. The obtained supernatant was then centrifuged at 100,000 g for 5h. The final pellet, containing the 20S proteasomes was resuspended in buffer (pH 7.5) containing 50 mM Tris-HCl, 5 mM MgCl₂, and 20% glycerol. The protein content of the proteasome preparation was determined based on Bio-Rad Protein Assay (Bio-Rad, Veenendaal, the Netherlands). The proteolytic activity of the 20S proteasomes was determined by measuring the activity against the fluorogenic substrates succinyl-leu-leu-val-tyr-7-amido-4-methylcoumarin (LLVY) and N-carbenzoxymethyl-leu-leu-glu-7-amido-4-methylcoumarin (LLE) (Sigma-Aldrich, Zwijndrecht, the Netherlands). These substrates are preferentially hydrolyzed by the chymotrypsin-like and peptidylglutamyl-peptide hydrolyzing peptidase activities of the 20S proteasome, respectively ². To measure proteolytic activity, 15 µg proteasome extract was added to 60 µL of medium containing 62.5 mM tris-HCl, 12.5 mM MgCl₂, 1.25 mM 1,4-dithiothreitol, 0.01 U apyrase, and 100 µM of LLVY or 375 µM of LLE. The reaction took place at 37°C for 45 min. The peptidase activity was determined by measuring the generation of the fluorogenic cleavage product (methylcoumarylamide) at 380 nm excitation wavelength and 440 nm emission wavelength

continuously with a spectrophotometer. Standard curves were established for the fluorogenic product, and peptidase activity was expressed as picomoles per microgram protein per minute. Addition of the proteasome inhibitor MG132 to the reaction resulted in complete inhibition of methylcoumarylamide production, indicating successful isolation of proteasomes without the presence of significant amounts of other proteases. For a representative activity vs time plot, see figure E1.

MAFbx and MURF-1 mRNA determination with real-time quantitative PCR

Total RNA was extracted from diaphragm samples using Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. The extracted RNA was dissolved in diethylpyro-carbonate (DEPC)-treated water and the concentration was determined by spectroscopy at 260 nm using the Ultrospec 1000 UV/Visible Spectrophotometer (Pharmacia Biotech, Foster City, CA). Total RNA was then reverse transcribed into cDNA using 50 ng of total RNA in a 20µl reaction volume by using SuperScript™ Reverse Transcriptase (Invitrogen, Carlsbad, CA). Quantitative PCR was performed in a total reaction volume of 25 µl per reaction. The 25 µl reaction mixture contained 12.5 µl of a SYBR green mix (Bio-Rad, Salt Lake City, UT), 10 pmol of each forward and reverse primer, 1µl cDNA and nuclease-free water to make up the reaction volume. Specific primers were selected using express software (Applied Biosystems, Foster City, CA). Forward and reverse oligonucleotides used were as following: MAFbx, 5'-CATCCTTATGTACTGGTCCA-AAGA-3' and 5'-ATCCGATACACCCACATGTTAATG-3', MuRF-1, 5'-AACTTGGAGAAGCAGCTGATCTG-3' and 5'-TAGGGATTTGCAG-CCTGGAA-3'; Glyseraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-ATTCCACCCATGGCAAATTC-3' and 5'-AT-TCCACCCATGGCAAATTC-3'. These primers were synthesized by Sigma Genosys. PCR runs were performed in triplicate using MyiQ real time PCR detection system

(Bio-Rad, Salt Lake City, UT). Levels of MAFbx and MuRF-1 mRNA were normalized to that of GAPDH in arbitrary unit.

Western blot analysis

Diaphragm samples were homogenized in ~200 µl ice-cold buffer (pH 7.5), containing 50 mM Tris, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulphonylfluoride (PMSF), and protease inhibitor cocktail (Sigma-Aldrich, Zwijndrecht, the Netherlands), by means of a Dounce homogenizer (Polytron, Rekken, the Netherlands). Homogenates were centrifuged at 10,000 x g, 4 °C for 10 min and the protein concentration of the resulting supernatants was determined using the Bio-Rad protein assay (Bio-Rad, Veenendaal, the Netherlands). Soluble proteins (10 µg) were subjected to routine Western blotting using 10% polyacrylamide sodium dodecyl sulfate (SDS)-gels and antibodies against the 20S proteasome subunit C8 (MCP72; mouse monoclonal; 1:10,000; Affiniti, Gorinchem, the Netherlands), and against myosin heavy chain (A4.1025; mouse ascites; 1:2500; Upstate, Lelystad, the Netherlands). After washing, blots were incubated with a horseradish peroxidase conjugated goat anti-mouse Ig (1:10,000, Pierce, Etten-Leur, the Netherlands). Chemiluminescence was performed using a ECLTM Western blotting analysis system (Amersham Biosciences, Roosendaal, Belgium). Protein bands were quantified using optical densitometry. Coomassie Blue-stained protein gels of the samples used for myosin heavy chain and C8 immunoblotting demonstrated equivalent amounts of protein loaded per lane.

Measurement of caspase-3 activity

Caspase-3 activity was determined as described by Du et al. ³, with minor modifications. Frozen diaphragm samples were pulverized and homogenized on ice in a buffer containing 100 mM HEPES (pH 7.5), 10% sucrose, 0.1 % Nonidet P-40, 10 mM dithiothreitol, and

protease inhibitor cocktail (Sigma-Aldrich, Zwijndrecht, the Netherlands). Homogenates were subjected to three cycles of freeze-thaw before centrifugation at 18,000 g for 30 min. The supernatant (85 ug protein) was added to reaction buffer consisting of 100 mM HEPES (pH 7.5), 10% sucrose, and 10 mM dithiothreitol. The fluorogenic substrate N-acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) was then added and the reaction was performed at 30°C for 60 min. The caspase-3 activity was determined by measuring the generation of the fluorogenic cleavage product (methylcoumarylamide) at 360 nM excitation wavelength and 460 nM emission wavelength with a spectrophotometer. Results were expressed as fluorogenic units per minute. Addition of N-Acetyl-Asp-Glu-Val-Asp-CHO (a caspase-3 inhibitor) to the reaction resulted in complete inhibition of methylcoumarylamide production. For a representative activity vs time plot, see figure E2.

Reference List

- E1. Hobler, S. C., A. Williams, D. Fischer, J. J. Wang, X. Sun, J. E. Fischer, J. J. Monaco, and P. O. Hasselgren. 1999. Activity and expression of the 20S proteasome are increased in skeletal muscle during sepsis. *Am.J.Physiol* 277:R434-R440.
- E2. Craiu, A., M. Gaczynska, T. Akopian, C. F. Gramm, G. Fenteany, A. L. Goldberg, and K. L. Rock. 1997. Lactacystin and clasto-lactacystin beta-lactone modify multiple proteasome beta-subunits and inhibit intracellular protein degradation and major histocompatibility complex class I antigen presentation. *J.Biol.Chem.* 272:13437-13445.
- E3. Du, J., X. Wang, C. Miereles, J. L. Bailey, R. Debigare, B. Zheng, S. R. Price, and W. E. Mitch. 2004. Activation of caspase-3 is an initial step triggering accelerated muscle proteolysis in catabolic conditions. *J.Clin.Invest* 113:115-123.

LEGENDS TO FIGURES

Figure E1. Representative plot of the proteolytic activity of isolated 20S proteasomes against the fluorogenic substrate LLVY vs time from a COPD and non-COPD patient. Addition of the proteasome-inhibitor MG132 almost completely abolished substrate cleavage.

Figure E2. Representative plot of the activity of diaphragm homogenates from a COPD and non-COPD patient against the caspase-3-specific fluorogenic substrate Ac-DEVD-AMC vs time. Addition of the caspase-3-inhibitor DEVD-CHO almost completely abolished substrate cleavage.

Figure E1.

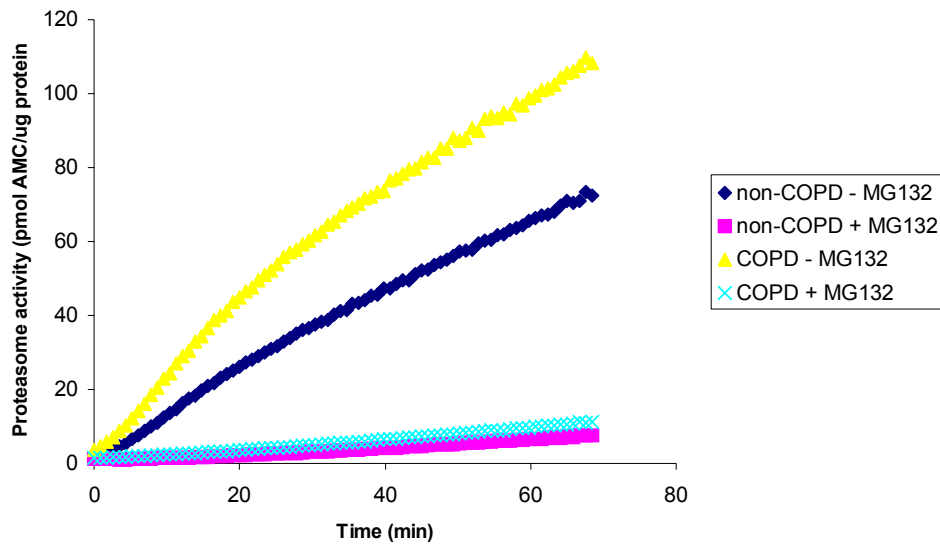


Figure E2.

