Proteasome inhibition improves diaphragm function in an animal model for COPD

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van Hees H, Ottenheijm C, Ennen L, Linkels M, Dekhuijzen R, Heunks L. Proteasome inhibition improves diaphragm function in an animal model for COPD. Am J Physiol Lung Cell Mol Physiol 301: L110–L116, 2011. First published April 1, 2011; doi:10.1152/ajplung.00396.2010.—Diaphragm muscle weakness in patients with chronic obstructive pulmonary disease (COPD) is associated with increased morbidity and mortality. Recent studies indicate that increased contractile protein degradation by the proteasome contributes to diaphragm weakness in patients with COPD. The aim of the present study was to investigate the effect of proteasome inhibition in diaphragm function and contractile protein concentration in an animal model for COPD. Elastase-induced emphysema in hamsters was used as an animal model for COPD; normal hamsters served as controls. Animals were either treated with the proteasome inhibitor Bortezomib (iv) or its vehicle saline. Nine months after induction of emphysema, specific force-generating capacity of diaphragm bundles was measured. Proteolytic activity of the proteasome was assayed spectrophotometrically. Protein concentrations of proteasome, myosin, and actin were measured by means of Western blotting. Proteasome activity and concentration were significantly higher in the diaphragm of emphysematous hamsters than in normal hamsters. Bortezomib treatment reduced proteasome activity in the diaphragm of emphysematous and normal hamsters. Specific force-generating capacity and myosin concentration of the diaphragm were reduced by ~25% in emphysematous hamsters compared with normal hamsters. Bortezomib treatment of emphysematous hamsters significantly increased diaphragm-specific force-generating capacity and completely restored myosin concentration. Actin concentration was not affected by emphysema, nor by bortezomib treatment. We conclude that treatment with a proteasome inhibitor improves contractile function of the diaphragm in emphysematous hamsters through restoration of myosin concentration. These findings implicate that the proteasome is a potential target of pharmacological intervention on diaphragm weakness in COPD.

Recent studies demonstrated that the concentration of contractile proteins in muscle fibers from the diaphragm of patients with COPD is significantly lower compared with non-COPD subjects (31, 33). This loss of contractile proteins provides an additional explanation for the development of diaphragm weakness in patients with COPD. Loss of contractile protein is accompanied by activation of the proteolytic ubiquitin-proteasome pathway, suggesting enhanced myosin degradation in the diaphragm of patients with COPD (32). Although these data imply a prominent role for the ubiquitin-proteasome pathway in COPD-induced diaphragm weakness, this was not specifically examined in previous studies. Therefore, the aim of the present study was to investigate the effect of proteasome inhibition on function and contractile protein concentration of the diaphragm in COPD. Bortezomib is one of the most specific and potent proteasome inhibitors presently available (2, 19). Although bortezomib has been studied in different experimental settings, its approval for clinical use in humans is at this moment restricted to treat patients with specific hematological malignancies (13, 35). Therefore, we decided to use an animal model for COPD to study the effect of bortezomib treatment on diaphragm function and contractile protein concentration. Elastase-induced emphysema in hamsters is a frequently used animal model for COPD because it resembles pulmonary hyperinflation, one of the key features of COPD (42). As in patients with COPD, pulmonary hyperinflation shortens the diaphragm of emphysematous hamsters. The hamster diaphragm adapts to this shorter length by removing sarcomeres in series; it is yet uncertain whether this occurs in patients with COPD as well (7). Nevertheless, we and others have demonstrated that the diaphragm of emphysematous hamsters produces less force even when measured at optimal muscle length and corrected for muscle cross-sectional area (16, 24, 39). This suggests that, like in patients with COPD, loss of contractile proteins contributes to diaphragm weakness in emphysematous hamsters. Accordingly, we hypothesized that bortezomib improves force-generating capacity through restoration of contractile protein concentration in the diaphragm of emphysematous hamsters.

MATERIALS AND METHODS

Animal model. Emphysema was induced as described previously (16, 39). Briefly, adult male outbred Syrian golden hamsters (~100 g) were intratracheally instilled with elastase (porcine pancreatic elastase, 18 U/100 g body wt; Sigma Aldrich, Zwijndrecht, the Netherlands). As a control group hamsters were instilled with an equal volume of saline (0.9%, 0.5 ml/100 g body wt). Verification and severity of emphysema was evaluated at the end of the experiments by...
estimating total lung volume on the basis of fluid displacement measurements, as described previously (16). This study was approved by the Animal Ethics Committee, Radboud University Nijmegen, The Netherlands.

**Treatment with proteasome inhibitor.** Nine months after elastase or saline instillation, animals were randomly assigned to bortezomib treatment or vehicle. Bortezomib (Velcade; Millennium Pharmaceuticals, Cambridge, MA) was injected intravenously (1.3 mg/kg ip) and mechanically ventilated. After a combined thoracotomy/laparotomy, the diaphragm muscle and adherent ribs were quickly excised, rinsed in saline to eliminate excessive blood contamination, and divided in parts. One part was snap-frozen in liquid nitrogen and stored at −80°C for later biochemical analyses, and the other parts were prepared for contractile measurements.

**Bundle contractility.** Diaphragm muscle-bundle contractile properties were measured as described previously (16, 39). Briefly, from the central costal region of the right and the left hemidiaphragms, one rectangular bundle from each was dissected parallel to the long axis of the muscle fibers. The bundles were mounted vertically in separate tissue baths containing Krebs solution, maintained at 26°C, and perfused with a 95% O2-5% CO2 gas mixture (pH 7.4). The costal margin of the diaphragm bundle was clamped to a micromanipulator for adjustment of length, and the central tendon of the diaphragm bundle was attached to a force transducer (model 308B; Cambridge Technologies, Cambridge, MA). The muscle bundle was stimulated directly by using platinum plate electrodes placed in close apposition of the bundle. Stimuli were applied with a pulse duration of 0.2 ms and train duration of 400 ms. Muscle preload force was adjusted until optimal fiber length (L0) for twitch force was achieved. After 10 min of thermodilution, maximal isometric-force generation was measured at a stimulation frequency of 100 Hz. After each experiment, muscle-bundle length and weight were determined. Cross-sectional area was calculated by dividing diaphragm strip weight (in g) by strip length (in cm) and multiplying it by specific density (1.056). Specific force was expressed as Newtons per cross-sectional area (in N/cm2).

**Proteasome activity.** Previous studies have shown that bortezomib mainly inhibits proteolytic activities of the chymotrypsin-like and peptidylglutamyl hydrolase (PDGH)-like sites but hardly affects the proteolytic activity of the trypsin-like site (18). Accordingly, we measured the proteolytic activities of the chymotrypsin-like and PDGH-like sites. Proteolytic activity was determined as described previously (32, 41). In short, 20S proteasomes were isolated from solubilized diaphragm samples in ice-cold buffer (pH 7.5) containing 50 mM Tris-HCl, 5 mM MgCl2, 250 mM sucrose, 1 mM 1,4-dithiothreitol, 0.2 mM phenylmethylsulphonylfluoride, and protease inhibitor cocktail (Sigma Aldrich). After three sequential centrifugations, the supernatants were centrifuged at 10,000 g, 4°C for 10 min. For analysis of phosphorylated Akt level diaphragm samples were homogenized in 100 volumes 62.5 mM Tris-HCl (pH 6.8), 0.5% SDS, 10% glycerol, 50 mM dithiothreitol, 0.01% Brome phenol blue, and protease and phosphatase inhibitor cocktails (Sigma Aldrich). Homogenates were centrifuged at 10,000 g, 4°C for 10 min. For determining actin concentration, diaphragm samples were homogenized in 10 volumes of ice-cold buffer, 125 mM Tris, pH 6.4, 10% glycerol, 4% SDS, 4 M urea, and 10% 2-mercaptoethanol, boiled for 3 min, and subsequently centrifuged for 3 min at 9,500 g, 20°C, according to Anderson et al. (3). Supernatants were loaded on 5% SDS-polyacrylamide gels to separate proteins. Gels were silver stained according to the procedure described by Oakley et al. (28). Protein bands were quantified by densitometer imaging (Genetools, Syngene, Cambridge, UK).

**Western blotting.** Proteasome concentration, Akt phosphorylation, and actin concentration were measured by means of Western blotting. For determination of proteasome concentration, 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 PMSF, and protease inhibitor cocktails (Sigma Aldrich). Homogenates were centrifuged at 10,000 g, 4°C for 10 min. For analysis of phosphorylated Akt level diaphragm samples were homogenized in 100 volumes 62.5 mM Tris-HCl (pH 6.8), 0.5% SDS, 10% glycerol, 50 mM dithiothreitol, 0.01% Brome phenol blue, and protease and phosphatase inhibitor cocktails (Sigma Aldrich). Homogenates were centrifuged at 10,000 g, 4°C for 10 min. For determining actin concentration, diaphragm samples were homogenized in 100 volumes of ice-cold buffer, 125 mM Tris, pH 6.4, 10% glycerol, 4% SDS, 4 M urea, and 10% 2-mercaptoethanol, boiled for 3 min, and subsequently centrifuged for 3 min at 9,500 g. After protein-concentration determination of the resulting supernatants, soluble proteins were subjected to routine Western blotting using polyacrylamide SDS-gels and specific antibodies [anti-20S proteasome subunit C8 antibodies from Affiniti, Gorinchem, the Netherlands (no. PBW110, diluted 1:10,000); anti-Akt and anti-phospho-Akt (Ser473) antibodies from Cell signaling Technology, Beverly, MA (no. 9272 and no. 9271, both diluted 1:1,000); anti-actin (CADOH terminus specific) from Sigma-Aldrich (no. A2055, diluted 1:500)]. After being washed, blots for proteasome concentration and phosphorylated Akt levels were incubated with a horseradish peroxidase-conjugated antibody (Pierce, EttenLeur, The Netherlands) for subsequent chemiluminescent detection. Protein bands were quantified using optical-densitometry software (GeneTools, Syngene, Cambridge, UK). For determination of actin concentration, blots were incubated with IR Dye-680-labeled goat anti-rabbit secondary antibody (LiCor, Bad Homburg, Germany) and subsequently scanned and quantified using an Infrared Laser Scanner and software (Odyssey; LiCor Biosciences, Lincoln, NE). Equal loading of the samples was confirmed by Coomassie blue staining of the gels and by Ponceau S staining of the blots.

**Data treatment and statistical analysis.** Because of a limited amount of diaphragm tissue per animal, not all analyses could be performed on tissue obtained from one animal although there is extensive overlap. Numbers of animals used for one analyses are mentioned in the legend of each figure. All data are presented as means ± SE. Differences between groups were analyzed with a one-way ANOVA. Student-Newman-Keuls post hoc testing was performed on data from emphysematous hamster saline vs. normal hamster saline, emphysematous hamster saline vs. emphysematous hamster bortezomib, and normal hamster saline vs. normal hamster bortezomib groups. P levels of <0.05 were considered significant.

**RESULTS**

**Animal characteristics.** Instillation with elastase resulted in severe pulmonary emphysema, as indicated by ~90% increase...
of lung volume ($P < 0.001$; Table 1). Body weight was lower in emphysematous hamsters than in normal hamsters ($P < 0.05$; Table 1). Bortezomib treatment did not affect the severity of emphysema, as indicated by unaltered lung volume. Increase of body weight during treatment period was not significantly different between groups.

**Proteasome activity and concentration.** Emphysema increased proteolytic activity of chymotrypsin-like sites and PDGH-like sites in proteasomes of the hamster diaphragm by $\sim 20\%$ ($P < 0.01$, Fig. 1A and $P < 0.05$, Fig. 1B). As expected, bortezomib treatment reduced proteolytic activities of both proteasomal sites in the diaphragm of emphysematous hamsters ($P < 0.001$) and normal hamsters ($P < 0.01$). Proteasome activity was analyzed in isolated proteasomes, indicating that proteolytic activity per proteasome is increased. To assess whether emphysema or bortezomib also affect the amount of proteasomes, we additionally measured muscle proteasome concentration in diaphragm homogenates. Proteasome concentration was higher in diaphragm from emphysematous hamsters compared with normal hamsters ($P < 0.001$, Fig. 2). Bortezomib treatment did not affect proteasome concentration in the diaphragm of emphysematous and normal hamsters.

**Diaphragm-specific force-generating capacity.** Maximal specific force generation was $\sim 25\%$ lower in diaphragm bundles from emphysematous hamsters compared with normal hamsters ($P < 0.001$, Fig. 3). Treatment of emphysematous hamsters with bortezomib significantly improved maximal specific force generation of the diaphragm ($P < 0.05$). Bortezomib treatment

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### Table 1. Animal characteristics of normal and emphysematous hamsters treated with proteasome inhibitor bortezomib or the vehicle saline

<table>
<thead>
<tr>
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<th>NH Saline (n = 20)</th>
<th>NH Bortezomib (n = 22)</th>
<th>EH Saline (n = 21)</th>
<th>EH Bortezomib (n = 22)</th>
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<tr>
<td>BW preinstillation, g</td>
<td>96 ± 2</td>
<td>94 ± 2</td>
<td>95 ± 3</td>
<td>94 ± 2</td>
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<tr>
<td>BW pretreatment, g</td>
<td>148 ± 2</td>
<td>142 ± 2</td>
<td>132 ± 2*</td>
<td>136 ± 2*</td>
</tr>
<tr>
<td>BW end treatment, g</td>
<td>151 ± 2</td>
<td>146 ± 2</td>
<td>132 ± 2*</td>
<td>137 ± 3*</td>
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<tr>
<td>Total lung volume, ml</td>
<td>11.1 ± 0.2</td>
<td>10.4 ± 0.3</td>
<td>21.0 ± 0.4*</td>
<td>21.2 ± 0.6*</td>
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Data are presented as means ± SE. BW, body weight; NH, normal hamster; EH, emphysematous hamster. *$P < 0.05$ vs. NH saline.

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Fig. 1. Chymotrypsin-like (A) and peptidylglutamyl hydrolase (PDGH)-like (B) proteolytic activity of isolated proteasomes from the diaphragm of normal hamsters (NH) and emphysematous hamsters (EH) treated with bortezomib or the vehicle saline ($n = 15–19$ animals per group). *$P < 0.01$ vs. NH saline, †$P < 0.001$ vs. EH saline. AMC, aminomethylcoumarin.

Fig. 2. Top: quantification of proteasome concentration in diaphragm of NH and EH treated with bortezomib or the vehicle saline ($n = 16–19$ animals per group). Bottom: representative Western blot. *$P < 0.001$ vs. NH saline.

Fig. 3. Maximal specific force-generating capacity of diaphragm bundles from NH and EH treated with bortezomib or the vehicle saline ($n = 18–20$ animals per group). *$P < 0.001$ vs. NH saline, †$P < 0.05$ vs. EH saline.
higher in diaphragm of emphysematous hamsters compared with normal hamsters ($P < 0.05$, Fig. 6, A and B). Bortezomib treatment did not significantly affect phosphorylated Akt and total Akt levels in the diaphragm of emphysematous and normal hamsters.

**DISCUSSION**

Recent studies from our group revealed that loss of contractile proteins, in particular myosin, plays a prominent role in diaphragm weakness in patients with COPD (29, 31–33). In these patients, loss of myosin was associated with activation of the ubiquitin-proteasome pathway. In the present study, similar observations were made in an animal model for COPD, i.e., enhanced proteolytic activity of the proteasome and loss of the contractile protein myosin. To follow-up on these findings, we studied the effect of proteasome inhibition on functional and biochemical characteristics of the diaphragm. The most important results of the present study are that treatment with a proteasome inhibitor restores myosin concentration and contractile function of the diaphragm in emphysematous hamsters. These findings implicate that diaphragm weakness in this animal model of COPD results from increased myosin degradation by the proteasome.

**Contractile protein degradation.** Numerous studies have demonstrated that the ubiquitin-proteasome pathway plays a major role in skeletal muscle wasting (12, 17, 23, 25, 38).

**Fig. 4.** Top: quantification of myosin concentration in diaphragm of NH and EH treated with bortezomib or the vehicle saline ($n = 17–20$ animals per group). Bottom: representative silver-stained gel. *$P < 0.05$ vs. NH saline, †$P < 0.05$ vs. EH saline.
However, few studies focused on specific loss of core myofilament proteins, such as myosin and actin. Muscle fiber-specific force generation strongly depends on myosin content (14), which underscores the clinical importance to investigate the fate of myosin during skeletal muscle wasting. Solomon and Goldberg (37) described 14 years ago that isolated proteasomes are able to degrade myosin in vitro. However, to conclusively establish that myosin is degraded by the proteasome in vivo, the availability of clinically saved proteasome inhibitors was indispensable. In a recent study, we indeed demonstrated that in vivo proteasome inhibition restored myosin concentration in diaphragm muscle fibers from animals with heart failure (40). Likewise, in the present study, we show that in vivo proteasome inhibition restores myosin concentration in an animal model for COPD. These data prove that myosin is degraded by the proteasome in vivo. Clinically more relevant is the finding that myosin recovery partially restores diaphragm contractile performance. This, not only indicates that the proteasome degrades myosin, but also suggests that the proteasome degrades myosin molecules that are functionally not compromised. The recovery of myosin is, however, not proportional to the restoration of diaphragm-specific force generation, i.e., myosin recovery is more pronounced than functional recovery. These findings suggest that the proteasome degrades both functional and dysfunctional myosin, and accordingly proteasome inhibition spares functional and dysfunctional myosin proteins. This is in line with the concept that the proteasome plays an indispensible role in recognition and subsequent degradation of dysfunctional proteins but is involved in the turnover of functional proteins as well (23, 34). In contrast to loss of myosin, we did not find a significant loss of actin, which indicates that the proteolytic machinery specifically targets myosin. Such specific loss of myosin has been reported in other diseases associated with muscle wasting, such as cancer (1, 6) and acute quadriplegic myopathy (27). It has been proposed that contractile proteins need to be released from the myofilaments before degradation by the proteasome because the proteasome is unable to degrade intact myofilaments (37). Recent publications suggest that release of myosin and actin is enforced by independent mechanisms. Disassembly of the thick filament and subsequent release of myosin is effectuated by the E3-ligase MuRF1 (8), whereas caspase-3 disassembles the thin filament by cleaving actin (11). According to a previous study (9), we did not find enhanced caspase-3 activities in the diaphragm of emphysematous hamsters (data not shown), which explains the lack of actin loss. Because no antibodies or Q-PCR primers are presently available to detect MuRF1 protein or mRNA in hamster tissue, we were unable to examine whether myosin loss in the present study was accompanied by increased MuRF1 expression. Interestingly to mention, however, is that bortezomib treatment has been shown to reduce MuRF1 levels in experimental models of heart failure (40) and burn injury-induced muscle wasting (22). Therefore, the recovery of myosin observed in the present study might be mediated by a direct effect of bortezomib on proteasomal proteolysis but also by lowering MuRF1 expression.

Interestingly, although bortezomib treatment of normal hamsters did reduce proteasome activities (Fig. 1), this did not increase myosin concentration or specific force-generating capacity of the diaphragm. These data are in line with previous investigations, which showed unaltered muscle weights and

Fig. 6. A, top: quantification of Akt phosphorylation in the diaphragm of NH and EH treated with bortezomib or the vehicle saline (n = 7 animals per group). Bottom: representative Western blot. *P < 0.05 vs. NH saline. B, top: quantification of total Akt in the diaphragm of NH and EH treated with bortezomib or the vehicle saline (n = 7 animals per group). Bottom: representative Western blot. *P < 0.05 vs. NH saline.
fiber cross-sectional areas in bortezomib-treated control animals (4, 20, 40), and indicates that bortezomib treatment is unlikely to induce muscle fiber hypertrophy in healthy animals.

Akt and protein turnover. Protein concentration is the net result of protein synthesis and degradation, and protein loss reflects an imbalance between these two. During skeletal muscle wasting, increased protein degradation is commonly accompanied by reduced protein synthesis. A primary role in regulating these two processes simultaneously has been ascribed to protein kinase B/Akt (26). When Akt is dephosphorylated, it inhibits protein synthesis and translation initiation by downstream signaling via glycogen synthase kinase-3β and mammalian target of rapamycin. At the same time, dephosphorylated Akt promotes protein degradation by enhancing expression of atrogene, like ubiquitin ligases. Thus dephosphorylation of Akt leads to protein loss by both promoting proteolysis and inhibiting protein synthesis. Unexpectedly, results from this study show increased phosphorylated Akt levels in the diaphragm of emphysematous hamsters, suggesting that protein synthesis is being promoted rather than being inhibited. These data are in line with previous observations from Doucet et al. (10), who found elevated levels of phosphorylated Akt in atrophied peripheral muscles of patients with COPD. The authors interpreted their data as a failed attempt of the muscle to compensate for protein loss. Our present data support that notion because, despite Akt activation, contractile protein loss occurs in the diaphragm of emphysematous hamsters. In addition, promotion of protein synthesis together with increased proteolysis reflects enhanced protein turnover. Treatment with bortezomib reduced proteasomal proteolysis in both normal and emphysematous hamsters; however, the high levels of phosphorylated Akt in the emphysematous hamsters were not affected by bortezomib. Consequently, protein synthesis is still being promoted in the diaphragm of bortezomib-treated emphysematous hamsters compared with normal hamsters. This could explain the finding that bortezomib treatment significantly increased myosin concentration in emphysematous hamsters but did not display a significant effect on myosin concentration in normal hamsters. These data are in line with previous studies, where bortezomib treatment increased muscle mass in diseased animals but not in healthy animals (4, 20, 40). We propose that, at least in the present study, enhanced protein turnover in the diseased diaphragm could explain this observation.

Clinical implications. Respiratory failure is a prominent manifestation in patients with severe COPD and follows from the incapacity of the respiratory muscles to meet the increased respiratory demand (21). Patients with COPD with weakened respiratory muscles are more prone to develop respiratory failure (5), which increases their mortality rate (15, 43). No proven pharmacological therapy is presently available to modulate respiratory muscle function in these patients. Results from previous studies on diaphragm biopsies from patients with COPD suggested that loss of contractile protein degradation might be a potential target for pharmaceutical intervention (29–33). The present study provides the, so far lacking, evidence that treatment with a clinically available proteasome inhibitor attenuates diaphragm weakness in an animal model for COPD. The present findings demonstrate that modulation of proteasome activity is indeed a potential new target for treatment of diaphragm weakness in COPD. However, the application of bortezomib in COPD is at this moment strictly experimental. Because the proteasome fulfills basic housekeeping cell functions, extensive research on side effects should warrant the therapeutic applicability of proteasome inhibitors in COPD. Finally, the present study indicates that protein synthesis is promoted in the diaphragm of emphysematous hamsters. This finding needs to be confirmed in human diaphragm biopsies because it would imply that protein synthesis is not an appropriate potential target for the development of new therapies.

In conclusion, the present study indicates that increased proteasomal degradation of myosin contributes to diaphragm weakness in an animal model for COPD. Treatment with a proteasome inhibitor partly recovers diaphragm-specific force-generating capacity. These data identify the proteasome as a potential therapeutic target to restore respiratory muscle function in COPD.

DISCLOSURES

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

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