Mechanical ventilation induces alterations of the ubiquitin-proteasome pathway in the diaphragm

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DeRuisseau, Keith C., Andreas N. Kavazis, Melissa A. Deering, Darin J. Falk, Darin Van Gammeren, Tossaporn Yimlamai, George A. Ordway, and Scott K. Powers. Mechanical ventilation induces alterations of the ubiquitin-proteasome pathway in the diaphragm. J Appl Physiol 98: 1314–1321, 2005. First published November 19, 2004; doi:10.1152/japplphysiol.00993.2004.—Prolonged mechanical ventilation (MV) results in diaphragmatic atrophy due, in part, to an increase in proteolysis. These experiments tested the hypothesis that MV-induced diaphragmatic proteolysis is accompanied by increased expression of key components of the ubiquitin-proteasome pathway (UPP). To test this postulate, we investigated the effect of prolonged MV on UPP components and determined the trypsin-like and peptidylglutamyl peptide hydrolyzing activities of the 20S proteasome. Adult Sprague-Dawley rats were assigned to either control or 12-h MV groups (n = 7/group). MV animals were anesthetized, tracheostomized, and ventilated with room air for 12 h. Animals in the control group were acutely anesthetized and not exposed to MV. Compared with controls, MV animals demonstrated increased diaphragmatic mRNA levels of two ubiquitin ligases, muscle atrophy F-box (+8.3-fold) and muscle ring finger 1 (+19.0-fold). However, MV did not alter mRNA levels of 14-kDa ubiquitin-conjugating enzyme, polyubiquitin, proteasome-activating complex PA28, or 20S α-subunit 7. Protein levels of 14-kDa ubiquitin-conjugating enzyme and proteasome-activating complex PA28 were not altered following MV, but 20S α-subunit 7 levels declined (−17.7%). MV increased diaphragmatic trypsin-like activity (+31%) but did not alter peptidylglutamyl peptide hydrolyzing activity. Finally, compared with controls, MV increased ubiquitin-protein conjugates in both the myofibrillar (+24.9%) and cytosolic (+54.7%) fractions of the diaphragm. These results are consistent with the hypothesis that prolonged MV increases diaphragmatic levels of key components within the UPP and that increases in 20S proteasome activity contribute to MV-induced diaphragmatic atrophy and proteolysis.

respiratory muscle; proteolysis; proteasome activity

MECHANICAL VENTILATION (MV) is a clinical intervention for patients who are unable to maintain adequate alveolar ventilation. For many patients, an unfortunate consequence of prolonged MV is the ensuing development of respiratory muscle atrophy and decreased ability to generate force (8). Inactivity of the diaphragm is hypothesized to be a vital component by which MV leads to the development of respiratory muscle weakness (1, 26). Lack of diaphragmatic contractile activity during controlled MV leads to increased oxidative injury (40, 46), decreased rates of protein synthesis (39), and enhanced rates of proteolysis (40); each of these factors is known to contribute to both muscle contractile dysfunction and atrophy (3, 25). Importantly, increased proteolysis is considered to be the major factor responsible for decreased skeletal muscle mass following disuse (24).

Three major proteolytic systems exist in skeletal muscle: 1) lysosomal, 2) Ca2+-dependent, and 3) ATP-dependent ubiquitin proteasome pathway (UPP) (12, 43). In this regard, the UPP is the major proteolytic system responsible for skeletal muscle myofibrillar protein degradation during disuse (12) and other catabolic states, including sepsis, fasting, cancer, metabolic acidosis, and burn injury (16, 24). Protein degradation by this multicomponent proteolytic pathway is accomplished primarily through a two-step process that involves substrate recognition (ubiquitin conjugation cascade) and protein degradation by the 26S proteasome (27). Formation of ubiquitin-protein conjugates occurs through the coordinated action of a three-enzyme system (32, 33). The ubiquitin-activating enzyme (E1) utilizes ATP-derived energy to form a covalent link with a ubiquitin protein (44). The ubiquitin is subsequently transferred to a ubiquitin-conjugating enzyme (E2), which may act in concert with a ubiquitin ligase (E3) to ultimately link the ubiquitin molecule with the substrate protein (16, 24). The ubiquitination process continues until a ubiquitin chain is formed on the substrate protein that signals degradation by the 26S proteasome in an ATP-dependent process (27).

Previous work in our laboratory has demonstrated that MV is associated with increased chymotrypsin-like (CT-L) activity of the 20S proteasome and an enhanced rate of proteolysis in the diaphragm (6, 40). However, it is unclear if prolonged MV promotes increased expression of key UPP components during the diaphragm. Moreover, it is unknown if prolonged MV elevates the trypsin-like (T-L) and peptidylglutamyl peptide hydrolyzing (PGPH) activities of the 20S proteasome. Therefore, the objectives of this experiment were twofold: 1) to investigate the effects of prolonged MV on the expression of key elements within the UPP in the diaphragm; and 2) to determine the impact of MV on diaphragmatic T-L and PGPH activities of the 20S proteasome. We employed 12 h of MV, as this period of time has been shown to significantly upregulate proteasome activity in the diaphragm (6). Based on reports using other models of disuse muscle atrophy (22, 23, 30), we formulated the working hypothesis that prolonged MV would promote the following changes in the diaphragm: 1) increased mRNA
levels of UPP components involved in ubiquitin-protein conjugation, including 14-kDa E2 (E2\textsubscript{14k}), polyubiquitin (pUb), muscle atrophy F-box (MAFBx/Atrogin-1), muscle ring finger 1 (MuRF1), and proteasome activity [proteasome-activating complex PA28 (PA28), 20S α-subunit 7 (C8)]; 2) increased levels of ubiquitin-protein conjugates of cytosolic and myofibrillar proteins; 3) increased E2\textsubscript{14k}, PA28, and C8 protein levels; and 4) increased T-L and PGPH activities of the 20S proteasome.

**METHODS**

**Experimental Animals**

These experiments were approved by the University of Florida animal use committee and followed the guidelines for animal experiments established by the American Physiological Society. Healthy, young adult (female, 4 mo old) Sprague-Dawley rats were individually housed and fed rat chow and water ad libitum and were maintained on a 12:12-h light-dark cycle for 2 wk before initiation of these experiments.

**Experimental Design**

Protocol for control animals. The animals in the control group (n = 7) were free of intervention before measurement of diaphragmatic gene expression and biochemical properties. That is, these animals were not mechanically ventilated or exposed to long-term anesthesia before study. Animals in the control group received an intraperitoneal (IP) injection of pentobarbital sodium (60 mg/kg body wt). After a surgical plane of anesthesia was achieved, the diaphragm was rapidly removed, and segments from the ventral costal region were rapidly frozen in liquid nitrogen and stored at −80°C for subsequent analysis.

Protocol for mechanically ventilated animals. All surgical procedures were performed using aseptic techniques. Animals randomly selected for MV (n = 7) were anesthetized with an IP injection of pentobarbital sodium (60 mg/kg body wt). After reaching a surgical plane of anesthesia, the animals were tracheostomized and mechanically ventilated using a volume-driven small-animal ventilator (Harvard Apparatus, Cambridge, MA) for 12 h. The ventilator delivered all breaths; hence, this mode of ventilation (i.e., controlled MV) resulted in complete diaphragmatic inactivity. The tidal volume was established at 0.55 ml/100 g body wt with a respiratory rate of 80 breaths/min. This respiratory rate was selected to mimic the breathing frequency of adult rats at rest. Additionally, positive end-expiratory pressure of 1 cmH\textsubscript{2}O was used throughout the protocol.

An arterial catheter was inserted into the carotid artery for constant measurement of blood pressure and periodic blood sampling for analysis of arterial pH and blood gases. Arterial blood samples (~100 μl/sample) were removed during the first and last hour of MV and analyzed for arterial PCO\textsubscript{2}, PO\textsubscript{2}, and pH using a blood-gas analyzer (Instrumentation Laboratories, model 1610). Before analysis, the analyzer was calibrated using standardized gases and pH solutions. Also, a venous catheter was inserted into the jugular vein to permit the infusion of isotonic saline and pentobarbital sodium when necessary. Anesthesia was maintained over the entire period of MV by continuous infusion of pentobarbital sodium (~10 mg/kg body wt/h). Throughout the experiments, technicians continuously monitored the level of anesthesia in MV animals by several methods (i.e., monitoring blood pressure and corneal/lid reflexes). Body temperature was maintained at ~37°C by use of a recirculating heating blanket. Additionally, heart rate and electrical activity of the heart were monitored via a lead II ECG using needle electrodes placed subcutaneously.

Throughout the period of MV, animals received enteral nutrition using the AIN-76 rodent diet with a nutrient composition of 15% proteins, 35% lipids, 50% carbohydrates, and vitamins and minerals (Research Diets, Brunswick, NY). Our planned feeding schedule was designed to provide an isocaloric diet with the nutrients administered every 2 h via a gastric tube; the total daily administration of 69 ml is equivalent to 69 kcal/day. Furthermore, body fluid homeostasis was maintained via the administration of 2.0 ml kg\textsuperscript{-1} h\textsuperscript{-1} intravenous electrolyte solution. Continuing care during MV included expressing the bladder, removing airway mucus, lubricating the eyes, rotating the animal, and passive movement of the limbs. This care was maintained throughout the experimental period at hourly intervals. Finally, repeated intramuscular injections of glycopyrrolate (0.04 mg kg\textsuperscript{-1} h\textsuperscript{-1}) were employed to reduce airway secretions. On completion of MV, segments of the ventral costal diaphragm were removed, rapidly frozen in liquid nitrogen, and stored at −80°C for subsequent analysis. To minimize the possible influence of circadian rhythm on gene expression levels, animals were euthanized at the same time (±1 h) each day (20).

**Biochemical Measurements**

Western blot analysis. A section (50–75 μg) of the costal diaphragm was homogenized and assayed to quantitatively determine the levels of ubiquitinated proteins, E2\textsubscript{14k}, PA28, and C8. Samples were homogenized 1:10 (5 mM Tris-HCl, pH = 7.5; 5 mM EDTA) and centrifuged at 1,500 g for 10 min (4°C). The supernatant (cytosolic fraction) was saved, and the pellet (myofibrillar fraction) was washed three times (5 mM Tris-HCl, pH = 7.5; 5 mM EDTA, pH = 8.0, 1.0% Triton X-100) and suspended in buffer (8.0 M urea; 50 mM Tris-HCl, pH = 7.5). The cytosolic fraction was centrifuged at 10,000 g for 10 min (4°C) followed by an additional spin of the supernatant at 100,000 g for 1 h (4°C). Protein content of the cytosolic and myofibrillar fractions was assessed by the method of Bradford (Sigma, St. Louis, MO). Proteins (50 μg) from the cytosolic and myofibrillar fractions were then individually separated by polyacrylamide gel electrophoresis via 4–15% gradient (cytosolic fraction) or 7.5% (myofibrillar fraction) polyacrylamide gels containing 0.1% SDS. After electrophoresis, the proteins were transferred to nitrocellulose membranes (100 V for 3 h). Membranes were stained with Ponceau S and visually inspected for equal protein loading and transfer. The membranes were then washed and blocked in PBS-Tween buffer containing 5.0% skim milk and 0.05% Tween for 2 h and subsequently incubated with a primary antibody directed against ubiquitin (Santa Cruz Biotechnology, Santa Cruz, CA), E2\textsubscript{14k}, PA28 α-subunit, or C8 (Boston Biochem, Cambridge, MA). Primary antibodies were diluted 1:500 (ubiquitin) or 1:1,000 (E2\textsubscript{14k}, PA28, and C8) in blocking buffer (8.0 M urea; 50 mM Tris-HCl; pH = 7.5) and applied to the membranes overnight at 4°C. This step was followed by incubation with a horseradish peroxidase-antibody conjugate (1:5,000) directed against the primary antibody for 1 h. The membranes were then treated with chemiluminescent reagents (luminol and enhancer) and exposed to light-sensitive film. Images of these films were captured and analyzed by using computerized image analysis (Gel Doc 2000, BioRad, Hercules, CA).

20S proteasome activity. The in vitro T-L and PGPH activities of the 20S proteasome were measured fluorometrically in crude extracts by following the release of free 7-amido-4-methyl-coumarin (AMC) from synthetic substrates (BioMol International, Plymouth Meeting, PA) by a modification of the method of Stein et al. (41). The proteasome substrates used were butyloxycarbonyl-Leu-Arg-Arg-AMC and benzyl oxyacyarbonyl-Leu-Leu-Glu-AMC for the measurement of T-L and PGPH activities, respectively. Ten micrograms of protein (cytosolic fraction) were reacted with the respective synthetic peptide substrates in a reaction mixture containing 50 mM Tris-HCl and 1 mM dithiothreitol. One aliquot from each sample was incubated with 10 μM MG132 (Sigma), an inhibitor of the T-L and PGPH proteasome activities (11), whereas the other aliquot was not incubated with the inhibitor. Release of AMC from the synthetic substrate in the presence of MG132 indicates the action of nonproteasomal proteases. Samples were incubated for 30 min at 37°C before the addition of substrate. The change in fluorescence was measured at an
excitation wavelength of 380 nM and emission of 460 nM. The difference between the activities of the proteasome with and without inhibitor was used as the 20S proteasome activity.

Molecular Measurements

Isolation of total RNA. A portion of the costal diaphragm (~60 mg) was homogenized in 1.5 ml of TRIzol reagent (Invitrogen, Carlsbad, CA) and processed according to the manufacturer’s instructions. Samples were homogenized with a Polytron blade homogenizer at medium speed and centrifuged at 12,000 g for 10 min (4°C) to remove insoluble material. The RNA portion was transferred to a new tube and extracted with 120 μl of bromochloroethane. Samples were vortexed briefly and centrifuged at 13,000 g for 20 min (4°C). Following transfer of the aqueous phase to a new tube, RNA was precipitated with one volume of isopropanol and washed twice with two volumes of 75% ethanol. The RNA was pelleted via centrifugation and resuspended in RNase-free water (Sigma). Concentration and purity of the extracted RNA were measured spectrophotometrically at 260 and at 280 nm in 1X Tris-EDTA (TE) buffer (Promega, Madison, WI). The integrity of the extracted total RNA was verified by gel electrophoresis of 1-μg RNA on a 1% agarose bromide-stained Tris-borate-EDTA gel. Total RNA was stored at −80°C.

Reverse transcription and cDNA quantification. Reverse transcription was performed using the Superscript III First-Strand Synthesis System for RT-PCR (Life Technologies, Carlsbad, CA), according to the manufacturer’s instructions. Reactions were carried out using 5 μg of total RNA and 2.5 μM oligo(dT)20 primers. First-strand cDNA was subsequently treated with 2 units of RNase H. Following the addition of 2 μl of GlycoBlue coprecipitant (Ambion, Austin, TX) to the RT product (21 μl), first-strand cDNA was cleaned of RNA and unincorporated nucleotides by treatment with an RNase cocktail (Ambion), brought to a volume of 100 μl with water, and applied to a NucAway spin column (Ambion). Samples were then mixed with phenol-chloroform-isomyl alcohol (pH = 7.9), and the aqueous phase recovered using a 1.5-ml heavy-phase lock gel (Eppendorf, Hamburg, Germany). The cDNA was precipitated by adding one volume of 5.0 M NH4OAc and two volumes of 100% EtOH and was stored at −20°C overnight. Following centrifugation at 13,000 g for 20 min (4°C), the cDNA was washed with two volumes of 75% EtOH, centrifuged at 13,000 g for 10 min (4°C), and resuspended in 50 μl of 1X TE buffer. The cDNA was subsequently quantified using the Oligreen ssDNA Quantitation Reagent and Kit, according to the manufacturer’s instructions (Molecular Probes, Eugene, OR).

Real-time quantitative PCR. The probes for all genes consisted of TaqMan 5'-labeled FAM reporters and 3' nonfluorescent quenchers. Quantitative real-time PCR was performed by using the ABI Prism 7000 Sequence Detection System (ABI, Foster City, CA). Primers and probes for MuRF1, MAFBx, E214k, C8, and β-glucuronidase were obtained from Applied Biosystems (Assays-on-Demand). The sequences used by the manufacturer in the design of primers and probes from this service are proprietary and are, therefore, not reported. However, the context sequences (i.e., the nucleotide sequence surrounding the probe) are available and consist of the following: MuRF1, 5'-AATGCTCAATGCAGCGTACACAA-3'; MAFbx, 5'-CCTGGAAGGGCACTGACCATCCATG-3'; E214k, 5'-ATCCAAATGTTGATGCTGACCGCAG-3'; C8, 5'-GTAAGTTAAAA-AAGTTGCAAATAATAA-3'; and β-glucuronidase, 5'-TACTCTCAGGACGGTATGCGCCACA-3'. The probes and PA28x primers and probes were obtained from Applied Biosystems (Assays-by-Design). Primer and probe sequences for pUb and PA28x were as follows: forward, 5'-ACCTCCCTGTATTACACATCTCAAC-3'; reverse, 5'-CGGCTACGGTCT-TCAGAAA-3'; and probe, 5'-CCTGCCACCTGTGCTCCTC-3'. Primer and probe sequences for PA28x are as follows: forward, 5'-GCTTCACAACCGCATACCATAGA-3'; reverse, 5'-TGCGGATAATCACCCACATGAG-3'; and probe, 5'-CTTGGCTGTTTGGCC-3'. Each 25-μl PCR reaction, performed in duplicate, contained 3.0 ng of cDNA template. Gene expression was calculated by using the relative standard curve method as described in the ABI, User Bulletin number 2. β-Glucuronidase was selected as the appropriate normalizer because the expression of this gene in the diaphragm is not significantly altered by MV (P = 0.76).

Statistical Methods

Comparisons between groups for each dependent variable measured were made by a one-way ANOVA. Significance was established at P < 0.05. Data are reported as means ± SE.

RESULTS

Systemic and Biological Response to MV

The animal body weights did not differ (P > 0.05) between the control (276.4 ± 7.0 g) and MV (pre-MV = 271.6 ± 7.5 g; post-MV = 272.0 ± 7.0 g) groups. These results confirm that our program of enteral nutrition and rehydration was adequate in the MV animals. Cardiovascular homeostasis during the experimental period was monitored via heart rate and systolic blood pressure. Heart rate (~360 beats/min) and mean blood pressure (>90 mmHg) were normal and well maintained during MV, i.e., the heart rate and blood pressure did not significantly decrease (P > 0.05) during the experiments. Blood-gas homeostasis and pH were maintained during MV, as previously described (9, 35, 40, 46). Because sepsis is associated with diaphragmatic contractile dysfunction, strict aseptic techniques were followed throughout the experiments. Our results indicate that the animals in our analysis did not develop infections during MV. This statement is supported by the observation that microscopic examination of blood revealed no detectable bacteria, and postmortem examination of the lungs (visual) and peritoneal cavity (visual) displayed no detectable abnormalities. The colonic temperature of all MV animals remained constant at 37 ± 0.5°C during the experiments. Furthermore, MV animals were afebrile during the investigation. Collectively, these results indicate that the MV animals used in this analysis were free of significant infection.

Effect of MV on mRNA Levels of UPP Components

Table 1 contains mRNA fold change values for genes assessed by real-time PCR; these values were calculated by dividing the average linear, normalized mRNA input values for the MV group by the control group. MV resulted in significant elevations in the mRNA levels of two skeletal muscle E3, MAFbx (+8.3-fold), and MuRF1 (+19.0-fold). A significant

<table>
<thead>
<tr>
<th>Gene GenBank mRNA</th>
<th>Fold (MV/Control)</th>
<th>P Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>E214k</td>
<td>M62388, AF144083</td>
<td>1.0 ± 0.17</td>
</tr>
<tr>
<td>MAFbx</td>
<td>AY059628</td>
<td>8.3 ± 0.39</td>
</tr>
<tr>
<td>MuRF1</td>
<td>AY059627, BC061824</td>
<td>19.0 ± 0.27</td>
</tr>
<tr>
<td>pUb</td>
<td>D16554</td>
<td>1.4 ± 0.20</td>
</tr>
<tr>
<td>PA28x</td>
<td>D45249</td>
<td>1.7 ± 0.15</td>
</tr>
<tr>
<td>C8</td>
<td>M58593</td>
<td>1.1 ± 0.21</td>
</tr>
</tbody>
</table>

*β-Glucuronidase Y00717, M13962

Values are means ± SE; n = 7/group. MV, mechanical ventilation; E214k, 14-kDa ubiquitin-conjugating enzyme; MAFbx, muscle atrophy F-box; MuRF1, muscle ring finger 1; pUb, polyubiquitin; PA28x, proteasome activator PA28 complex; C8, 20S α-subunit 7. *P value calculated by one-way ANOVA on linear, normalized (β-glucuronidase) input values.
increase in PA28α mRNA level was also detected following 12 h of MV. No significant differences in the mRNA levels of E214k, pUb, or C8 were detected in the MV group compared with controls.

Ubiquitin-Protein Conjugates and Levels of PA28, E214k, and C8

Figure 1 contains representative Western blots, illustrating the degree of protein ubiquitination in cytosolic and myofibrillar diaphragm fractions of control and MV samples. Ubiquitin-protein conjugates in myofibrillar (Fig. 1B) and cytosolic (Fig. 1D) diaphragm fractions are illustrated next to respective membranes stained with Ponceau S (Fig. 1, A and C). Western blots were quantified by using densitometry in which the analysis of ubiquitin-protein conjugates included all bands (i.e., the entire lane) for each sample. Expressed relative to control values, MV significantly increased the level of ubiquitin-protein conjugates of both cytosolic and myofibrillar fractions by 54.7 and 24.9%, respectively ($P < 0.05$). Figure 2 contains representative Western blots for E214k, PA28, and C8. Expressed relative to control, C8 protein levels were significantly decreased following MV (−17.7%; $P < 0.05$). No significant changes in the protein levels of E214k or PA28 were observed following 12 h of MV.
Overview of Principle Findings

These experiments provide new and important information regarding the impact of prolonged MV on the UPP in the diaphragm. Our findings expand on our laboratory’s previous observations of elevated rates of protein synthesis and increased activity of the 20S proteosome in the diaphragm following MV (6, 40). Specifically, these new data reveal that MV promotes an increase in the level of ubiquitin-protein conjugates of both the cytosolic and myofibrillar fractions of the diaphragm and elevates diaphragmatic mRNA levels of two important skeletal muscle proteins. Our laboratory has recently discovered that controlled MV results in increased oxidized levels of myofibrillar, but not cytosolic, proteins in the diaphragm (40, 46). In the context of MV-induced diaphragmatic atrophy, protein oxidation is significant because oxidized proteins are sensitive to proteolytic degradation (14). Therefore, the increased level of protein ubiquitination of soluble proteins in the diaphragm during MV may reflect a greater degree of protein turnover, as evidenced by increased protein ubiquitination, and may explain, at least in part, the lack of protein carbonyl accumulation in the cytosolic fraction (46).

Increased ubiquitin-protein conjugation during skeletal muscle disuse has been associated with elevated mRNA levels of

Effect of MV on In Vitro 20S Proteasome Activities

Our laboratory has previously observed increased in vitro CT-L activity in the diaphragm following 12 (6) and 18 h (40) of MV. Therefore, in these experiments, we determined the in vitro activity of the 20S proteosome by examining the T-L and PGPH activities. Twelve hours of MV significantly increased the T-L activity (+31.0%), but did not significantly alter PGPH activity (−5.1%) (Fig. 3).

DISCUSSION

MV-induced Alterations of UPP Components in the Diaphragm

Previous work from our laboratory has revealed large increases in the rates of proteasome-mediated protein degradation in conjunction with increased CT-L activity of the 20S proteosome in the diaphragm of animals exposed to prolonged MV (6, 40). The present investigation advances these earlier reports by investigating MV-induced changes in key components of the UPP in the diaphragm in an effort to better understand the involvement of this proteolytic system in MV-induced diaphragmatic atrophy.

The present data reveal that MV is associated with increased ubiquitination of proteins in both the cytosolic and myofibrillar diaphragm fractions. The increased level of ubiquitin-protein conjugates in the myofibrillar fraction (24.9%; P < 0.05) is in accordance with the degree of ubiquitination observed in the gastrocnemius following 3 days of denervation (45). The myofibrillar fraction, comprising 50–60% of the muscle protein, has been shown to be the principle protein component targeted by the UPP in various models of muscle wasting, including disuse atrophy (12). The targeting and subsequent degradation of myofibrillar proteins by the UPP likely contributes to the atrophy and weakness of skeletal muscle following periods of disuse. The soluble proteins of the cytosolic fraction also demonstrated an increased level (54.7%; P < 0.05) of ubiquitin-protein conjugates within the diaphragm of MV animals. Our laboratory has recently discovered that controlled MV results in increased oxidized levels of myofibrillar, but not cytosolic, proteins in the diaphragm (40, 46). In the context of MV-induced diaphragmatic atrophy, protein oxidation is significant because oxidized proteins are sensitive to proteolytic degradation (14). Therefore, the increased level of protein ubiquitination of soluble proteins in the diaphragm during MV may reflect a greater degree of protein turnover, as evidenced by increased protein ubiquitination, and may explain, at least in part, the lack of protein carbonyl accumulation in the cytosolic fraction (46).
E2, E3, ubiquitin, and several components of the 26S proteasome (7, 23, 30, 43). The association between increased rates of proteolysis and mRNA of the UPP is suggestive of transcriptional regulation of this pathway. In support of this statement, one of the most dramatic changes observed in the diaphragm following MV was increased mRNA levels of the E3, MAE-fbx/Atrogin-1, and MuRF1 (7, 13). E3 include a family of proteins involved in the attachment of ubiquitin to protein substrates (27). In cell culture, overexpression of MAE-fbx/Atrogin-1 leads to a significant degree of atrophy in skeletal myotubes (7). Conversely, the prevention of increased expression of MAE-fbx/Atrogin-1 and MuRF1 through gene knockdown has been shown to attenuate denervation-induced muscle atrophy (7). Consistent with other models of muscle atrophy, our results demonstrate large increases in the diaphragmatic expression of these two E3, in conjunction with increased proteasome activity and atrophy in response to MV (40). It is evident, however, that not all mRNA of UPP components in the diaphragm are upregulated in response to MV, because no significant changes were observed in the mRNA levels of E2_1a, pUb, or C8. It is possible that 12 h of MV were not a sufficient stimulus to induce detectable alterations in the expression of these genes. Moreover, it cannot be ruled out that MV affected mRNA stability (18). It is apparent, however, that increased protein ubiquitination of the cytosolic and myofibrillar diaphragm fractions following 12 h of MV did not require increased mRNA expression of E2_1a, pUb, or C8.

**MV-induced Upregulation of Proteasome Activity**

A new finding in the present investigation is the observation of increased T-L, but not PGPH, proteasome activity in the diaphragm following 12 h of MV. In these studies, we utilized crude cytosolic extracts to reflect in vivo 20S proteasome activity. However, several endogenous factors have been shown to influence the activity of the proteasome, including the following: 1) increased substrate levels (21); 2) increased proteasome protein levels (10, 19); and 3) increased levels of proteasome regulatory complexes, PA700 and PA28 (i.e., specific activity) (19).

Although an increased level of protein ubiquitination may increase the amount of substrate for the 26S proteasome in vivo, this is not reflected in our in vitro assay of 20S proteasome activity. However, oxidatively modified proteins have been shown to be substrates for the 20S proteasome in a process that does not require ATP (15). Importantly, our laboratory has reported increased levels of oxidized proteins in the diaphragm following as little as 6 h of MV (46). Moreover, we have recently demonstrated the ability of Trolox, a water-soluble analog of vitamin E, to attenuate MV-induced proteasome activity (6). Hence, it is feasible that the accumulation of oxidized proteins in the diaphragm following MV may increase the amount of substrate for the 20S proteasome and stimulate in vitro activity.

An unexpected finding in our experiments was the −17.7% decrease in the amount of C8 protein levels in the diaphragms of animals exposed to MV, as no alteration in the muscle level of mRNA was detected. α-Subunits of the 20S core, which are structural and noncatalytic subunits, are commonly used to assess proteasome content (4, 17, 21). In our experiments, we chose to measure C8, as mRNA levels for this protein have been shown to increase in other models of muscle atrophy (5, 30). The decreased C8 levels could reflect an increased degradation of the subunit in response to MV (30) or a decrease in translational efficiency (19). Nonetheless, despite the small reductions observed in the amount of C8 protein, MV was still associated with a significant increase in T-L activity.

Important regulators of proteasome activity include the proteasome activators PA700 and PA28 (16). As mentioned previously, it is unlikely that the protein levels of PA700 in the diaphragm affected our in vitro assay of 20S proteasome activity, because these activities were not assayed in the presence of ATP (10, 19). However, because we did not examine PA700 subunits, we cannot exclude the possibility that changes in the amount of the PA700 complex may influence the in vivo degradation of ubiquitinated proteins. Unlike the PA700 regulatory unit, the PA28 regulatory complex directly affects the peptidase activity of the 20S proteasome in a process that does not require ATP (2, 36). In this regard, our data revealed no change in PA28 protein levels in the diaphragm following MV, despite a significant increase (+1.7-fold) in the amount of mRNA. It is unlikely, therefore, that levels of PA28 were responsible for the increased 20S proteasome activities in the diaphragm following MV.

**Critique of Experimental Model**

Because of the invasive nature of removing diaphragm samples, animal models must be used to study the effects of MV on respiratory muscle biochemistry. Considerations in the choice of an animal model include both practical considerations (e.g., size of the animal) and the applicability of the animal model to humans. In this regard, we chose the rat model for several reasons. First, adult rats are of adequate size to permit surgical procedures and removal of several arterial blood samples for blood-gas analysis during prolonged MV. Furthermore, and most importantly, human and rat diaphragms are similar in fiber-type composition, gross anatomical features, and function (31, 34).

We chose acutely anesthetized animals to serve as appropriate controls, as we have previously documented that the diaphragm of spontaneously breathing animals does not atrophy or demonstrate elevated rates of proteolysis (40). Finally, we chose pentobarbital sodium as the general anesthetic in these experiments because of direct evidence that this anesthetic does not promote oxidative injury in the diaphragm or negatively impact diaphragm atrophy or contractile function (35, 38, 46).

**Summary and Conclusions**

These experiments provide new and important information regarding the effects of prolonged MV on gene expression and protein levels of key components of the UPP in the diaphragm. Clinically, these findings are important as they identify potential mechanisms in which to target interventional strategies for preventing MV-induced diaphragmatic wasting in patients exposed to prolonged periods of MV. Nonetheless, the extent to which ubiquitin-protein conjugation contributes to in vivo protein degradation and functional properties such as contractile function remains unknown. Future studies should be directed toward uncovering signaling pathways that may be involved in the upregulation of diaphragmatic UPP activity in...
response to MV, including the roles of reactive oxygen species (14, 29) and the phosphatidylinositol 3-kinase/Akt pathway (28, 37, 42). Knowledge of these pathways as they pertain to UPP-mediated proteolysis would enhance the ability to effectively counteract this proteolytic system during MV-induced diaphragmatic atrophy and weakness.

REFERENCES


