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# Mechanical Ventilation–induced Diaphragmatic Atrophy Is Associated with Oxidative Injury and Increased Proteolytic Activity

Authors:

**R. Andrew Shanely**, Murat A. Zergeroglu, Shannon L. Lennon, Takao Sugiura, Tossaporn Yimlamai, Debbie Enns, Angelo Belcastro, and Scott K. Powers

Abstract

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# Mechanical Ventilation–induced Diaphragmatic Atrophy Is Associated with Oxidative Injury and Increased Proteolytic Activity

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**Prolonged mechanical ventilation (MV) results in reduced diaphragmatic maximal force production and diaphragmatic atrophy. We investigate the mechanisms responsible for MV-induced diaphragmatic atrophy, we tested the hypothesis that controlled MV results in oxidation of diaphragmatic proteins and increased diaphragmatic proteolysis due to elevated protease activity. Further, we postulated that MV would result in atrophy of all diaphragmatic muscle fiber types. Mechanically ventilated animals were anesthetized, tracheostomized, and ventilated with 21% O<sub>2</sub> for 18 hours. MV resulted in a decrease ( $p < 0.05$ ) in diaphragmatic myofibrillar protein and the cross-sectional area of all muscle fiber types (i.e., I, IIa, IIb/x, and IIc). Further, MV promoted an increase ( $p < 0.05$ ) in diaphragmatic protein degradation along with elevated ( $p < 0.05$ ) calpain and 20S proteasome activity. Finally, MV was also associated with a rise ( $p < 0.05$ ) in both protein oxidation and lipid peroxidation. These data support the hypothesis that MV is associated with atrophy of all diaphragmatic fiber types, increased diaphragmatic protease activity, and augmented diaphragmatic oxidative stress.**

**Keywords:** myosin heavy chain; oxidative stress; protein degradation; weaning

Mechanical ventilation (MV) provides a means of supporting blood gas homeostasis for patients who cannot maintain adequate alveolar ventilation. Unfortunately, prolonged MV (i.e., 3 days or more) is not without consequence because as many as 20% of patients experience difficulty in “weaning” from the ventilator (1). Although the underlying causes for weaning difficulties are not completely clear, respiratory muscle weakness due to atrophy and contractile dysfunction are potential mechanisms (2).

To date, only a few studies have investigated respiratory muscle function after controlled MV. In this regard, Le Bourdelles and coworkers (3) examined the effects of 48 hours of controlled MV on both atrophy and contractile properties in the rat diaphragm. The authors reported a significant reduction in isometric force generation and a decrease in both diaphragmatic mass (i.e., atrophy) and protein content (3).

Further, experiments performed in our laboratory have confirmed that as few as 18 hours of MV results in diaphragmatic contractile dysfunction (4) and atrophy (our unpublished observations). The mechanism(s) responsible for this atrophy are unknown and are the focus of the experiments described in this article.

Experiments investigating disuse locomotor skeletal muscle atrophy (e.g., hindlimb unloading) indicate that whereas all muscle fibers atrophy during prolonged periods of unloading, slow (i.e., Type I) muscle fibers are particularly susceptible to this type of atrophy (reviewed by Roy and coworkers [5]). In contrast, diaphragmatic inactivity induced by either unilateral denervation or tetrodotoxin blockade of nerve impulses results in atrophy of Type IIx and IIb fibers and hypertrophy of Type I and IIa fibers (6–8). At present, it is unclear which diaphragmatic fiber types are subject to atrophy during MV. It is also well known that locomotor muscle atrophy due to reduced use is associated with an increase in both oxidative stress (9) and protease-mediated protein degradation (10). In contrast, it is unknown whether prolonged MV results in increased protease activity and elevated protein degradation. Further, it is also unclear whether MV is associated with oxidative injury in the diaphragm. Therefore, on the basis of several voids in knowledge about the cellular effects of MV on the diaphragm, these experiments were designed to address the following questions.

## Question 1

Which diaphragmatic fiber types are subject to atrophy during MV? To address this question, immunohistochemical procedures were used to identify diaphragmatic fiber types and computerized image analysis was employed to determine the effect of MV on muscle fiber cross-sectional area. On the basis of preliminary data in our laboratory, we hypothesized that short-term controlled MV induces atrophy of all four muscle fiber types in the diaphragm.

## Question 2

Which proteolytic pathways are activated during MV? Two differing but complementary approaches were used to probe the activation of specific proteolytic pathways. First, we investigated the effects of specific calpain and proteasome inhibitors on the rates of protein breakdown in diaphragmatic strips *in vitro*. Further, we measured diaphragmatic calpain and proteasome activities. On the basis of preliminary experiments, we postulated that controlled MV increases diaphragmatic proteolysis by elevating both calpain and proteasome activities.

## Question 3

Does MV result in oxidative injury in the diaphragm? This is an important question relative to diaphragmatic atrophy because

oxidized proteins are associated with increased proteolysis (11–14). Therefore, tissue levels of protein carbonyls and 8-isoprostane were measured to determine whether MV results in diaphragmatic protein oxidation and lipid peroxidation, respectively. On the basis of preliminary data, we hypothesized that controlled MV increases oxidative stress in the diaphragm.

## METHODS

### Animals and Experimental Design

These experiments were approved by the University of Florida Animal Use Committee. Adult female Sprague-Dawley rats were randomly assigned to the following groups: (1) 18 hours of mechanical ventilation (MV) ( $n = 16$ ), (2) control (CON) (acute anesthesia, no MV;  $n = 16$ ), and (3) spontaneous breathing (SB) (anesthetized and spontaneously breathing for 18 hours;  $n = 6$ ).

### Mechanical Ventilation Protocol

Surgical procedures were performed using aseptic technique. After reaching a surgical plane of anesthesia (sodium pentobarbital, 50 mg/kg body weight, intraperitoneal), animals were tracheostomized and mechanically ventilated (control mode) using a volume-driven ventilator (Columbus Instruments, Columbus, OH). The tidal volume was about 1 ml/100 g of body weight, with a respiratory rate of 80 breaths/minute, and a positive end-expiratory pressure (PEEP) of 1 cm H<sub>2</sub>O.

The carotid artery was cannulated for measurement of arterial blood pressure, pH, and blood gases. A total of three arterial blood samples (about 100  $\mu$ l/sample) were removed from animals at 5- to 6-hour intervals to determine arterial pH and blood gas tensions, using an electronic blood gas analyzer (Model 1610; Instrumentation Laboratory, Lexington, MA). The jugular vein was also cannulated for the infusion of saline and sodium pentobarbital (about 10 mg/kg body weight per hour). Body temperature was maintained at about  $37 \pm 1^\circ\text{C}$ . Heart rate was monitored via a lead II electrocardiograph.

Continuing care during the experiment (both MV and SB animals) included expressing the bladder, removing airway mucus, lubricating the eyes, rotating the animal, passive movement of the limbs, and enteral nutrition. After 18 hours of mechanical ventilation, the diaphragm was removed, rapidly dissected, frozen, and stored at  $-80^\circ\text{C}$ .

### Protocol for Control Animals

Control animals were free of intervention and were anesthetized (sodium pentobarbital, about 50 mg/kg, intraperitoneal). After reaching a surgical plane of anesthesia, the diaphragm was removed, dissected, frozen, and stored at  $-80^\circ\text{C}$ .

### Spontaneous Breathing Protocol

Animals were anesthetized (sodium pentobarbital, about 50 mg/kg, intraperitoneal) and a surgical plane of anesthesia was maintained with sodium pentobarbital (about 10 mg/kg every hour, intravenous). These animals were not mechanically ventilated and breathed spontaneously during this time. Spontaneously breathing animals received continuing care and enteral nutrition as described for the MV animals. After 18 hours of spontaneous breathing, the diaphragm was removed, dissected, frozen, and stored at  $-80^\circ\text{C}$ .

### Tissue Analysis

All morphometric (histochemical) and biochemical assays were conducted on the costal region of the diaphragm. Specifically, diaphragm samples were removed from the entire midcostal muscle spanning from the costal margin to the central tendon. Samples used for morphometric analysis were frozen (liquid nitrogen) at an unstressed length (15) and stored at  $-80^\circ\text{C}$ . Immunohistochemistry was performed to identify muscle fiber types and fiber cross-sectional area (CSA) as determined by computerized image analysis. Also, diaphragmatic myofibrillar protein concentration and total content were measured (16, 17) along with diaphragmatic water content.

As a marker of total *in vitro* protein degradation, the rate of tyrosine release from isolated diaphragm strips (about 40 mg/strip) was measured with and without specific proteolytic inhibitors (18, 19). Nonlysosomal and noncalpain proteolysis (i.e., ubiquitin-proteasome system)

was measured in the presence of the lysosomal and calpain inhibitor E-64d (Sigma, St. Louis, MO) (20, 21). Further, lysosomal and calpain proteolysis was measured in the presence of the proteasome inhibitor lactacystin (Boston Biochem, Boston, MA) (22, 23). Total calpain-like activity (24, 25) and activity of the 20S proteasome (26) were also determined in diaphragm samples.

Levels of lipid peroxidation (8-isoprostane) (ELISA; Cayman Chemical, Ann Arbor, MI) and protein oxidation (protein carbonyl content) (27) were measured to determine oxidative damage in the diaphragm.

Comparisons between groups were made by a one-way analysis of variance and a Tukey test was used post hoc.

## RESULTS

### Systemic and Biologic Response to Mechanical Ventilation

Because hypoxia, hypercapnia, and respiratory acidosis are known to induce diaphragmatic dysfunction, we monitored arterial blood pressures, pH, and the partial pressures of both CO<sub>2</sub> and O<sub>2</sub> during the 18 hours of MV in all MV animals. Arterial blood pressure and blood gas/pH homeostasis were well maintained during the period of MV. For example, arterial Po<sub>2</sub> ranged from 95 to 79 mm Hg, whereas arterial pH varied from 7.42 to 7.47 during the 18 hours of mechanical ventilation.

Sepsis is associated with diaphragmatic contractile dysfunction; therefore, aseptic techniques were used throughout the MV experiments. Our observations indicate that the animals did not develop infections during MV. Microscopic examination of blood at the conclusion of MV revealed no detectable bacteria and postmortem examination of the lungs (histological) and peritoneal cavity (visual) revealed no abnormalities. Finally, we did not observe a significant decrease in total body mass or locomotor muscle mass after prolonged MV (Table 1). This is an important observation because sepsis is associated with rapid decreases in both body and locomotor muscle mass. Collectively, these observations indicate that our animals did not develop sepsis during MV.

### Body Weight and Diaphragmatic Atrophy

Table 1 contains mean values ( $\pm$  SEM) for body weight, diaphragm mass, as well as soleus mass obtained from both control and mechanically ventilated animals. Note that 18 hours of MV resulted in a significant decrease ( $p < 0.05$ ) in both total and costal diaphragmatic mass. Although crural diaphragmatic mass tended to be lower in MV animals compared with control animals, this difference did not reach significance ( $p = 0.12$ ). As mentioned above, MV did not result in a loss of body mass or decrease soleus muscle mass (Table 1). These observations indicate that the mechanically ventilated animals received adequate nutrition and that the observed skeletal muscle atrophy was unique to the diaphragm.

### Fiber CSA

Immunohistochemical analysis revealed that fiber CSA did not differ in any fiber type between the control and spontaneously breathing animals. This indicates that exposure to 18 hours of sodium pentobarbital anesthesia does not result in diaphragmatic atrophy. In contrast, compared with control animals, 18 hours of MV induces significant myofiber atrophy as the CSA of all four fiber types was reduced ( $p < 0.05$ ) by MV (Figure 1). Interestingly, the Type II fibers atrophied to a greater extent than the Type I fibers,  $-24$  to  $-30\%$  versus  $-15\%$ , respectively. This disproportionate decrease in Type II fiber CSA resulted in an increase ( $p < 0.05$ ) in the percentage of total area occupied by the Type I fibers (Table 2). Figure 2 presents four light micrographs obtained from a control animal. Each section of the costal diaphragm was stained with a monoclonal antibody that reacts with a specific rat myosin heavy chain (MHC) type.

**TABLE 1. BODY AND MUSCLE WEIGHTS OF MECHANICALLY VENTILATED, SPONTANEOUSLY BREATHING, AND CONTROL ANIMALS**

	CON	SB	MV
Initial body mass, g	277.2 + 5.1	307.4 + 8.7*	290.1 + 9.1
Final body mass, g	277.2 + 5.1	302.1 + 8.4*	289.4 + 8.9
Total DIA mass, mg	1,056 + 16	1,162 + 38*	981 + 11* <sup>†</sup>
Total DIA mass/body mass <sup>†</sup> , mg/g	3.82 + 0.07	3.85 + 0.06	3.42 + 0.1* <sup>†</sup>
Costal DIA mass, mg	699 + 12	789 + 34*	648 + 7* <sup>†</sup>
Costal DIA mass/body mass <sup>†</sup> , mg/g	2.53 + 0.05	2.60 + 0.05	2.26 + 0.07* <sup>†</sup>
Crural DIA mass, mg	357 + 8	374 + 10	334 + 12 <sup>†</sup>
Crural DIA mass/body mass <sup>†</sup> , mg/g	1.29 + 0.3	1.24 + 0.05	1.16 + 0.05
Soleus mass, mg	131.8 + 8	129.8 + 5	140.7 + 6
Soleus mass/body mass <sup>†</sup> , mg/g	0.47 + 0.022	0.43 + 0.012	0.49 + 0.016

*Definition of abbreviations:* CON = control animals; DIA = diaphragm; MV = mechanical ventilation animals; SB = spontaneously breathing animals.

Values represent means + SEM.

\* Significantly different from control group,  $p < 0.05$ .

<sup>†</sup> Significantly different from spontaneously breathing group,  $p < 0.05$ .

<sup>†</sup> Mass values expressed as milligrams per gram were normalized to postexperiment body mass values.

### Measurement of Myofibrillar Protein Concentration and Water Content

Table 3 contains measurements of diaphragmatic protein concentrations and water content. Note that the protein and water content did not differ between the control and spontaneously breathing animals. In contrast, compared with control animals, 18 hours of MV resulted in significant reductions ( $p < 0.05$ ) in all measures of diaphragmatic protein. The concentrations of both myofibrillar protein and soluble protein decreased ( $p < 0.05$ ) by about 10%, resulting in a significant decrease ( $p < 0.05$ ) in the total protein concentration. Consistent with the loss of diaphragmatic mass was the reduction in total and myofibrillar protein content, reflecting an absolute loss of protein from the diaphragm. In addition, MV resulted in a mean increase (about 4%) in muscle water content. Note that this increase in diaphragmatic water content was observed in every experimental animal.

### MV, Protease Activity, and Proteolysis

Our results clearly indicate that both total calpain-like activity and the activity of the 20S proteasome are elevated by 18 hours of MV (Figure 3). Indeed, 18 hours of MV elevated calpain-like activity by about 128%. Similarly, MV resulted in a 470% increase in the activity of the 20S proteasome.

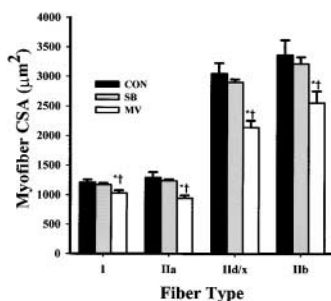
Whole muscle breakdown was determined by measuring the rate of tyrosine release from *in vitro* diaphragm strips. Compared with control animals, the rate of diaphragmatic catabolism was accelerated after 18 hours of MV as indicated by a 46% increase ( $p < 0.05$ ) in tyrosine release (Figure 4). Note that the rate of tyrosine release was not measured from diaphragms obtained

from the anesthetized, spontaneously breathing animals. The rationale for this exclusion was that the spontaneously breathing animals did not experience diaphragmatic atrophy, as indicated by the finding that fiber CSA, muscle wet weight, and muscle protein content did not differ ( $p > 0.05$ ) from the acutely anesthetized (control) animals.

Addition of the calpain blocker E-64d resulted in a significant decrease in diaphragmatic proteolysis in both the control and MV animals (Figure 4). Further, inhibition of proteasome activity by the addition of lactacystin resulted in a significant decline in the rate of diaphragmatic proteolysis in both control and MV animals. Collectively, these results confirm that MV-induced diaphragmatic proteolysis is mediated by both the calpain and proteasome systems.

### Measurement of Diaphragm Oxidative Injury

Two measures of oxidative stress were assessed to determine whether prolonged MV is associated with an increase in diaphragmatic oxidative damage. Compared with control animals, protein oxidation, as measured by protein carbonyl levels, and lipid peroxidation, as measured by 8-isoprostane concentration, were significantly increased by 18 hours of MV, 44 and 53%, respectively (Figures 5A and 5B). In contrast, no differences existed in protein carbonyl levels between control and spontaneously breathing animals. Note that 8-isoprostane was not measured from diaphragms obtained from the anesthetized, spontaneously breathing animals because the spontaneously breathing animals did not experience diaphragmatic atrophy (as mentioned above) and the protein carbonyl content did not differ from that of the acutely anesthetized (control) animals.



**Figure 1.** Diaphragmatic muscle fiber cross-sectional area (CSA) from control animals (CON), spontaneously breathing animals (SB), and animals exposed to 18 hours of mechanical ventilation (MV). Values represent means + SEM ( $n = 4$ /group). \*Significantly less ( $p < 0.05$ ) than control animals; <sup>†</sup>significantly less ( $p < 0.05$ ) than spontaneously breathing animals.

**TABLE 2. PERCENTAGE OF TOTAL CROSS-SECTIONAL AREA OCCUPIED BY EACH DIAPHRAGMATIC FIBER TYPE**

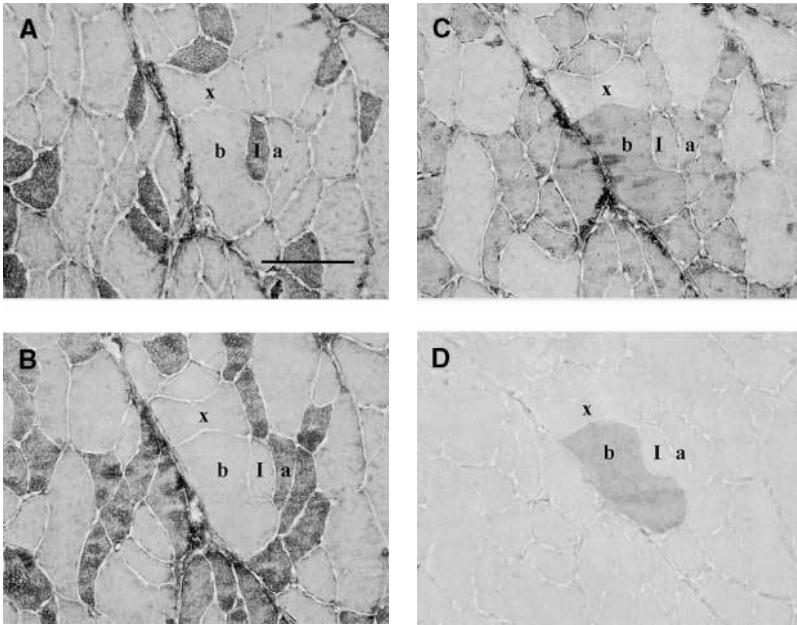
Group	Type I	Type IIa	Type IId/x	Type IIb
CON	24.6 + 1.1	22.7 + 1.5	42.9 + 1.9	9.8 + 1.3
SB	25.1 + 1.5	25.5 + 1.1	41.9 + 2.1	7.4 + 1.0
MV	29.5 + 0.5* <sup>†</sup>	24.3 + 2.4	38.1 + 2.1	8.1 + 0.3

*Definition of abbreviations:* CON = control animals; MV = mechanical ventilation animals; SB = spontaneously breathing animals.

Values represent mean percentages + SEM.

\* Significantly different from control group,  $p < 0.05$ .

<sup>†</sup> Significantly different from spontaneously breathing group,  $p < 0.05$ .



**Figure 2.** Photomicrographs illustrating the use of immunohistochemistry to identify costal diaphragm fiber types and determine fiber CSA in a control rat. Serial cross-sections of diaphragm muscle were immunohistochemically stained with (A) anti-Type I MHC (BA-D5), (B) anti-Type IIa MHC (SC-71), (C) anti-Type IIx MHC (BF-35), or (D) anti-Type IIb MHC (BF-F3). Type I MHC, I; Type IIa MHC, a; Type IIx MHC, x; Type IIb MHC, b. Scale bar in (A) represents 100  $\mu$ m.

## DISCUSSION

### Overview of Major Findings

These are the first experiments to examine the mechanism(s) responsible for MV-induced diaphragmatic atrophy. Our data support the supposition that MV is associated with increased proteolysis and atrophy of all diaphragmatic fiber types. Further, our data uphold the hypothesis that MV-induced proteolysis is associated with increased diaphragmatic calpain and 20S proteasome activity along with an increase in diaphragmatic oxidative stress. A detailed discussion of these data and the interpretations related to our hypotheses follow.

### MV-Induced Diaphragmatic Atrophy

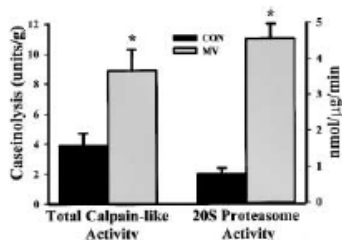
Importantly, our results indicate that no differences existed in any of our diaphragmatic measurements (e.g., diaphragmatic mass, fiber CSA, protein content) between the control (acute anesthesia) and the spontaneously breathing animals (18 hours of anesthesia). These observations reveal that sodium pentobarbital anesthesia is not responsible for the diaphragmatic proteolysis and atrophy associated with controlled MV.

Consistent with a previous report (3), our data indicate that controlled MV resulted in significant atrophy within the costal region of the rat diaphragm. Interestingly, the observed atrophy was unique to the diaphragm, as there was no loss of total body mass and no reduction in the mass of the soleus muscle. These results indicate that the removal of mechanical activity from the

chronically active diaphragm via controlled MV leads to rapid muscle atrophy.

We identified diaphragmatic MHC types by a multiple monoclonal antibody immunohistochemical approach and measured fiber CSA via computerized image analysis. Our results support the hypothesis that 18 hours of controlled MV promotes a significant reduction in the CSA of all four diaphragmatic MHC types. Interestingly, Type II fibers atrophied to a greater extent than Type I fibers. The fact that Type II fibers undergo the greatest degree of atrophy in the diaphragm during MV differs from investigations of locomotor muscle during periods of muscle disuse. Indeed, reports using the hindlimb suspension model of unloading rat skeletal muscles indicate that whereas Type II fibers suffer muscle atrophy during disuse, Type I fibers are preferentially susceptible to muscle atrophy during muscle unloading (reviewed in Roy and coworkers [5]).

The observation that MV results in diaphragmatic atrophy in all muscle fiber types also differs from several studies investigating diaphragmatic adaptation to inactivity. Specifically, two previous experiments reveal that 14 days of diaphragmatic inactivity, due to either bilateral denervation or tetrodotoxin blockade of nerve impulses, results in a selective atrophy of Type IIb and IIx fibers and a transient hypertrophy of both Type I and IIa fibers (6, 8). The biological mechanism(s) responsible for this variation in the diaphragmatic response to differing models of inactivity are unclear but could be related to differences in passive movement of the diaphragm between these experimental paradigms. For example, it has been shown that during controlled MV, the right and left hemidiaphragms undergo passive shortening during mechanical expansion of the lungs (28). In contrast, bilateral inactivity of the diaphragm, due to either nerve blockage or denervation, results in passive stretching of the inactivated diaphragm region during inspiration (7). This is significant because it has been postulated that the transient hypertrophy of Type I and IIa fibers in the rat diaphragm during bilateral denervation is due to increased protein synthesis induced by the passive stretch of the muscle during breathing (29, 30). Although this is an attractive explanation for these experimental differences, the direct effect of either passive stretch or passive



**Figure 3.** Effect of mechanical ventilation on total calpain-like activity and 20S proteasome activity in the diaphragm of control (CON) and mechanical ventilation (MV) animals. Values represent means  $\pm$  SEM ( $n = 4$ /group). \*Significantly greater than control,  $p < 0.05$ .

**TABLE 3. DIAPHRAGMATIC PROTEIN AND WATER CONTENT**

	CON	SB	MV
Total protein concentration, mg/g	237.2 + 5.1	239.6 + 4.7	213.2 + 9.4* <sup>†</sup>
Total protein content, <sup>†</sup> mg	165.6 + 3.6	163.7 + 3.2	138.2 + 6.2* <sup>†</sup>
Myofibrillar protein concentration, mg/g	128.2 + 2.5	136.1 + 2.3	115.3 + 5.2* <sup>†</sup>
Myofibrillar protein content, <sup>‡</sup> mg	89.5 + 1.6	93.1 + 1.5	74.8 + 3.4* <sup>†</sup>
Soluble protein concentration, <sup>§</sup> mg/g	108.9 + 2.8	103.5 + 4.5	97.8 + 4.4*
Soluble protein content, <sup>  </sup> mg	76.1 + 1.9	70.7 + 3.1	63.4 + 2.3*
Water content, %	74.3 + 0.4	74.4 + 1.1	77.4 + 0.5* <sup>†</sup>

*Definition of abbreviations:* CON = control animals; g = gram wet weight; MV = mechanical ventilation animals; SB = spontaneously breathing animals.

Values represent means + SEM. All measurements were made on the costal portion of the diaphragm.

\* Significantly different from control group,  $p < 0.05$ .

<sup>†</sup> Significantly different from spontaneously breathing group,  $p < 0.05$ .

<sup>‡</sup> Total protein content equals costal diaphragm mass (g) times costal protein concentration.

<sup>§</sup> Total myofibrillar protein content equals costal diaphragm mass (g) times costal myofibrillar protein concentration.

<sup>||</sup> Soluble protein content equals costal diaphragm mass (g) times costal soluble protein concentration.

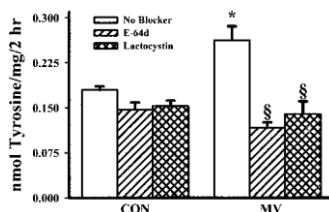
shortening on diaphragmatic adaptation to inactivity remains unknown; this is an interesting area for future research.

Concomitant with the MV-induced decrease in muscle fiber CSA was the loss of diaphragmatic protein. Compared with control diaphragms, 18 hours of MV was associated with a reduction in both diaphragmatic myofibrillar and soluble protein; this loss of protein was observed in both protein concentration and total protein content. In addition to the loss of protein, MV resulted in a 4% increase in diaphragmatic water content. The increase in muscular water content would explain the decrease in muscle protein concentration and is consistent with the increased water content that occurs in conjunction with muscular injury (31). Nonetheless, whether MV results in diaphragmatic injury cannot be determined from our current data.

Functionally, this MV-induced reduction in protein concentration could contribute to the observed reduction in diaphragmatic maximal specific force production after MV (3, 4). Theoretically, the reduction in myofibrillar protein concentration would result in fewer myosin cross-bridges per cross-sectional area of muscle and therefore less specific force generation. This is a testable hypothesis and is worthy of study.

### Mechanisms for MV-induced Atrophy

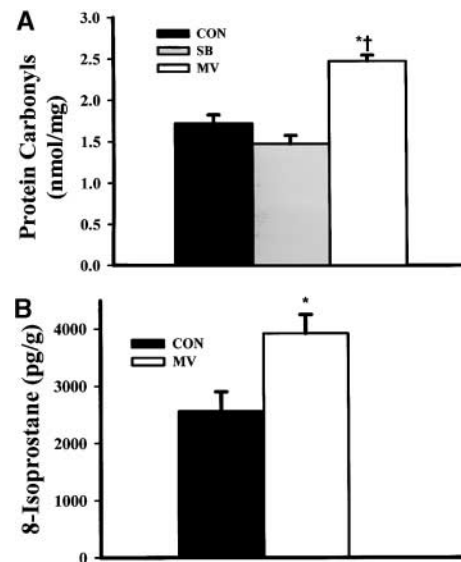
A reduction in skeletal muscle protein can occur as a result of a decreased rate of protein synthesis, an increased rate of protein degradation, or both. Previous studies investigating locomotor skeletal muscle atrophy during disuse indicate that this type of atrophy is due to both a reduced rate of protein synthesis and an increased rate of protein breakdown. Indeed, within hours of the onset of muscle disuse (i.e., hindlimb unweighting) the rate of muscle protein synthesis can decline by as much as 50%



**Figure 4.** Effect of mechanical ventilation on whole muscle protein catabolism as measured by the rate of tyrosine released from *in vitro* diaphragm strips per wet weight of muscle in 2 hours, and the impact of specific calpain (E-64d) and proteasome (lactacystin) inhibitors on the rates of diaphragmatic proteolysis. Control animals, CON; mechanical ventilation animals, MV. Values represent means + SEM (n = 4/group). \* Significantly greater than control value,  $p < 0.05$ .

<sup>§</sup> Significantly less than mechanical ventilation tyrosine release, under no blocker condition,  $p < 0.05$ .

(32). However, because the rate of protein synthesis was not measured in the present study, the relative contribution of decreased protein synthesis to MV-induced atrophy of the diaphragm cannot be quantified. Nonetheless, our results support the hypothesis that protein degradation is significantly accelerated after 18 hours of MV and that MV-induced diaphragmatic atrophy is due, in part, to an increased rate of protein degradation (Figure 4). To clarify the role that specific proteases play in this MV-induced proteolysis, we used complementary but differing experimental approaches that included measurements of *in vitro* protein breakdown with and without proteolytic inhibitors along with measurement of calpain and 20S proteasome activities. Our results indicate that both the calpain and 20S proteasome systems contribute to MV-induced diaphragmatic proteolysis. Indeed, compared with control animals, both 20S proteasome and calpain activities were elevated in diaphragms of MV animals (Figure 3). Furthermore, we observed a significant



**Figure 5.** Effect of mechanical ventilation on two measures of oxidative stress, protein carbonyl concentration (A), and total 8-isoprostane concentration in pg/g wet weight of muscle (B). Control animals, CON; spontaneously breathing animals, SB; mechanical ventilation animals, MV. Values represent means + SEM (n = 8/group). \* Significantly greater than control value,  $p < 0.05$ ; <sup>†</sup> significantly greater than spontaneously breathing animal value,  $p < 0.05$ .

reduction in the rate of tyrosine release during *in vitro* incubation of diaphragm strips from MV animals after the addition of either a proteasome inhibitor (lactacystin) or an inhibitor of both calpain and lysosomal proteases (E-64d). Collectively, these results provide strong support for the notion that both the calpain and proteasome systems contribute to MV-induced diaphragmatic proteolysis. Although it is possible that lysosomal proteases also contribute to MV-induced proteolysis in the diaphragm, previous experiments suggest that the contribution of cathepsins to muscle disuse atrophy is quantitatively small (10).

Finally, our results also support our hypothesis that MV results in an increase in diaphragmatic oxidative stress as indicated by the increase in the diaphragmatic content of both protein carbonyls and total 8-isoprostane. In the context of MV-induced diaphragmatic atrophy, an increase in protein oxidation is important because moderately oxidized proteins are more sensitive to proteolytic attack by proteases (11–14). Therefore, oxidative modification of proteins could accelerate protein degradation in the diaphragm during MV. Further, the ubiquitin–proteasome pathway is the proteolytic pathway implicated in the degradation of actin and myosin in muscle (10, 33), and this pathway is upregulated during periods of oxidative stress (34–36). An oxidative stress–mediated upregulation of the ubiquitin–proteasome pathway would lead to an increase in protein degradation and thus atrophy. Therefore, we postulate that oxidative stress may play an integral role in MV-induced atrophy of the diaphragm.

## Conclusion

Our findings clearly demonstrate that short-term controlled MV leads to rapid diaphragmatic atrophy. Indeed, after 18 hours of MV, diaphragmatic protein content and mass were significantly reduced and this was reflected by a decrease in the CSA of all four diaphragmatic MHC types. Further, oxidative stress was increased and total protein degradation was accelerated after MV. The MV-induced increase in diaphragmatic proteolysis was associated with an increase in both calpain and 20S proteasome activities. Future experiments should be directed toward understanding signaling pathways responsible for both the sequential activation of proteolytic systems and the production of oxidants within the diaphragm during periods of unloading due to MV.

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