Cumulative Effects of Aging and Mechanical Ventilation on In Vitro Diaphragm Function*

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Study objective: Unloading the diaphragm, via mechanical ventilation (MV), results in significant diaphragmatic atrophy, contractile dysfunction, and oxidative stress in young adult animals. Since aging increases skeletal muscle susceptibility to atrophy and injury, we tested the hypothesis that MV-induced diaphragmatic contractile dysfunction would be exacerbated in aging rats.

Methods: Fisher 344/Brown Norway hybrid rats (4 months old [young] and 30 months old [old]) were assigned to either control or MV groups. MV rats were anesthetized, tracheostomized, and ventilated with 21% O2 for 12 h. Arterial BP, pH, and blood gas homeostasis were maintained in the MV animals throughout the experimental period. Animals in the control group were acutely anesthetized, and the diaphragms were immediately removed. Muscle strips from the mid-costal diaphragm were removed from each experimental animal, and contractile properties were studied in vitro.

Results: Compared to young control animals, aging (old control animals) was associated with a 13% decrease in maximal isometric tension (24.5 N/cm² vs 21.3 N/cm²). Although, MV induced similar relative losses (24%) in diaphragmatic isometric tension in both young and old animals receiving MV, the combined effects of aging and MV resulted in a 34% decrement in diaphragmatic isometric tension compared to young control animals (24.5 N/cm² vs 16.1 N/cm²).

Conclusions: These data do not support the hypothesis that aging exacerbates the relative MV-induced impairment in diaphragmatic isometric tension. Nonetheless, the additive effects of aging and MV have dramatic effects on diaphragmatic force reserve. This could exacerbate weaning difficulties in older individuals receiving MV. (CHEST 2003; 124:2302–2308)

Key words: aging; artificial respiration; contractile function; diaphragm

Abbreviations: MV = mechanical ventilation; TPT = time-to-peak tension

Difficulty in weaning patients from mechanical ventilation (MV) is a serious clinical problem.1–4 Factors that influence the incidence of weaning problems include variables such as severity of illness, duration of MV, and age of the patient.4,5 We and others have demonstrated that controlled MV results in diaphragmatic weakness and atrophy.6–8 This is significant because diaphragmatic atrophy and weakness are major contributors to difficult weaning from MV.1,8–11 Indeed, weaning difficulties are commonly associated with respiratory muscle failure secondary to an inspiratory muscle force deficit and a reduction in inspiratory muscle endurance.2,12

Aging is associated with a loss of skeletal muscle mass and strength, referred to as sarcopenia. Additionally, compared to young adults, skeletal muscles from old animals are more susceptible to injury and atrophy.13,14 Further, we have shown that regeneration and recovery from injury is impaired or delayed in old skeletal muscle.15 Although the mechanisms responsible for these aging effects have not been fully elucidated, these observations suggest that the aging diaphragm may be more sensitive to the unloading caused by prolonged controlled MV. We have shown previously that antioxidant enzyme ac-

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Manuscript received January 22, 2003; revision accepted June 10, 2003.

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tivity is elevated in aging diaphragm muscle. Although this was interpreted to indicate increased production of free radicals in aging diaphragm, no age-related effects on direct indices of oxidative stress in diaphragm have been reported.

Unlike locomotor skeletal muscle, diaphragm mass is maintained with aging, probably due to the chronic activity of this muscle. However, maximal tetanic-specific tension in the aging rat diaphragm is reduced. Given this existing diaphragm weakness in senescent individuals, MV-induced diaphragmatic weakness could contribute to weaning problems in the elderly. In fact, age is an independent predictor of weaning difficulties in human patients following surgery, therefore, we tested the hypothesis that compared to young adult animals, aging would exacerbate MV-induced diaphragmatic contractile dysfunction.

Recent evidence suggests that oxidative stress contributes to the diaphragmatic contractile dysfunction following MV. Since oxidative stress has also been linked to aging, we tested the secondary hypothesis that markers of acute cellular oxidation (ie, glutathione depletion and accumulation of protein carbonyls) would be increased in aging diaphragms, and that MV-induced cellular oxidation would be exaggerated with aging.

**Materials and Methods**

**Animals**

Male Fisher 344/Brown Norway hybrid (F344xBN F1) rats, 4 months old and 30 months old, were obtained from the National Institute on Aging (Bethesda, MD), individually housed, and maintained on a 12 h/12 h light-dark cycle for 2 weeks before use. All rats were provided with food (American Institute of Nutrition 1993 recommended standard laboratory rodent diet) and water ad libitum. Male rats were chosen to avoid potential confounding effects of age-related changes in estrogen.

**Experimental Design**

Animals from the two age groups were randomly classified into two subgroups (n = 6 rats per group per age group): (1) control, and (2) mechanical ventilation (MV) for 12 h. Immediately following the 12-h treatment period, rats receiving MV were killed by an overdose of sodium pentobarbital and the diaphragms were quickly removed for analyses of mass, contractile function, protein content, markers of oxidative stress, and antioxidant capacity. The control rats were anesthetized and the diaphragms immediately removed for analysis. These experiments were approved by the Animal Care and Use Committee of the University of Florida.

**MV**

Animals in the MV groups were anesthetized with an injection of sodium pentobarbital (50 mg/kg body weight), tracheostomized, and received MV with a volume-cycled ventilator (Harvard Apparatus; Cambridge, MA). Heart rate and electrical activity of the heart were monitored via a lead-II ECG using needle electrodes placed subcutaneously. An arterial catheter was placed in the carotid artery for constant measurement of BP and periodic blood sampling (every 3 h) for analyses of arterial pH and blood gases. Arterial Po2, Pco2, and pH were maintained within the normal range (80 to 110 mm Hg, 30 to 40 mm Hg, and 7.35 to 7.45, respectively) by minor adjustments to tidal volume. Finally, a venous catheter was placed in the jugular vein for infusion of sodium pentobarbital (10 mg/kg/h) and fluids, when necessary. Body (rectal) temperature was measured hourly and maintained at 37 ± 1°C by use of a recirculating heating blanket. Body fluid homeostasis was maintained via the administration of 2 mL/kg/h IV electrolyte solution.

Animals received continuous monitoring during the MV period. Continuing care during MV included expressing the bladder, removal of airway mucus, lubricating the eyes, rotating the animal, and passive movement of the limbs. Details of our MV protocol have been described elsewhere.

**Notes on Choice of Ventilator and Anesthesia:** These experiments investigated the effects of controlled MV on the diaphragm by using volume-cycled ventilators. A major advantage of volume-cycled ventilators is that tidal volume remains relatively constant despite possible pathophysiologic changes (eg, airway obstruction due to mucus production). Further, volume-cycled ventilators are capable of maintaining a consistent fraction of inspired oxygen, which is important in maintaining blood gas homeostasis during MV. Volume ventilators have become widely used for human ventilatory support since their introduction in the 1950s.

We chose sodium pentobarbital as the general anesthetic because of our long experience with this type of barbiturate and the fact that Le Bourdelles et al have shown that the level of barbiturate required to maintain general anesthesia for 48 h in rodents does not alter locomotor muscle contractile and biochemical properties. We have demonstrated that 18- to 24-h spontaneous breathing under pentobarbital sodium anesthesia without MV has no effect on in vitro diaphragm contractile function and oxidative stress in rats. However, 18 to 24 h of spontaneous breathing under anesthesia does result in hypoxemia, hypercapnia, and mild acidosis likely due to hypoventilation from the reduced ventilatory drive. No differences in the diaphragmatic force-frequency response were noted between acutely anesthetized animals and long-term anesthesia of 18 to 24 h. Eighteen hours of spontaneous breathing under pentobarbital sodium anesthesia did not significantly alter diaphragmatic mass, fiber cross-sectional area, protein content, or markers of oxidative stress such as protein carbonyl content compared to acutely anesthetized controls. Our laboratory has also shown that sodium pentobarbital anesthesia is not responsible for the diaphragmatic proteolysis and atrophy induced by controlled MV. Therefore, in the present study, we are confident that changes in the diaphragm muscle after 12 h of treatment can be attributed exclusively to the MV protocol.

**Diaphragm Preparation and Contractile Measurements**

Contractile performance of the diaphragm was measured in vitro on costal diaphragm strips, as described previously by our laboratory. Briefly, the entire diaphragm was removed and placed in a dissecting chamber containing a Krebs-Henseleit solution maintained at 24 ± 0.5°C and equilibrated with a 95% O2/5% CO2 gas. A muscle strip was dissected out from the mid-ventral costal region for in vitro contractile measurements, while the remaining diaphragm was weighed and frozen for subsequent analytical measurements.
After a 15-min period of equilibration to the bath, the length of the in vitro strip was set at optimal length by systematically adjusting the length of the muscle using a micrometer while evoking twitch contractions (maximal monophasic pulse). Maximal tetanic force, force-frequency relationship (500-ms trains, 15 to 200 Hz, 120 V), and fatigue characteristics (250 ms-trains, 30 Hz, 120 V, 1 train/2 s for 30 min) were then measured as described previously.17

**Total and Myofibrillar Protein Measurements**

Myofibrillar protein was isolated from a portion of the costal diaphragm using a modification of the myofibril extraction technique described by Solaro et al22; protein concentration was then determined using the biuret technique. Briefly, muscle portions were scissors minced, homogenized using a glass-on-glass homogenizer in 4 mL of sucrose buffer (250 mM sucrose, 100 mM KCl, 5 mM ethylenediamine tetra-acetic acid, 20 mM Tris, pH 6.8), and centrifuged for 15 min at 2,500 g. The supernatant was discarded and the pellet suspended in a KCl buffer containing Triton X-100 to eliminate membrane adenosine triphosphatase components (175 mM KCl, 0.5% Triton X-100, 20 mM Tris, pH 6.8). This was centrifuged for 10 min at 2,500 g and the supernatant discarded. The pellet was again suspended in the KCl buffer containing Triton X-100 to eliminate membrane adenosine triphosphatase components (175 mM KCl, 0.5% Triton X-100, 20 mM Tris, pH 6.8). This was centrifuged for 10 min at 2,500 g and the supernatant discarded. The pellet was then determined using the biuret technique. Depletion of cellular glutathione content is a reliable index of acute oxidant stress because it returns rapidly to normal after the oxidant challenge is removed.23,24

**Glutathione Assay**

Total glutathione was measured in the costal diaphragm using a commercially available kit (Cayman Chemical; Ann Arbor, MI), according to the instructions of the manufacturer. Depletion of cellular glutathione content is a reliable index of acute oxidant production in skeletal muscle.22,23

**Assessment of Protein Oxidation in the Diaphragm**

To determine the extent of radical mediated oxidative damage in the diaphragm, portions of the costal diaphragm were analyzed for total protein oxidation. Protein oxidation was measured in the insoluble protein fraction of diaphragm homogenates via the protein carbonyl method described by Reznick and Packer.24

**Statistical Analysis**

Main effects were determined for the biochemical measures using 2 × 2 factorial analysis of variance. Where significant differences were found, Tukey HSD test was implemented post hoc. A 4 × 6 (group × stimulation frequency) analysis of variance with repeated measures on stimulation frequency was used to analyze the force-frequency data. Significance was established at p < 0.05.

**RESULTS**

**Body Mass, Muscle Mass, and Diaphragm Protein Contents**

Table 1 provides mean (± SEM) body mass, diaphragm mass, and diaphragm protein contents for each experimental group. Unlike humans, male rats continue to grow throughout adulthood as evidenced by the significantly larger body mass in the 30-month-old rats compared to the 4-month-old young adults. However, there was a corresponding increase in diaphragm mass in the aged rats, such that diaphragm mass to body mass ratio was not significantly different between young and old animals. Although basal metabolic rate relative to total body mass declines with age in the rat,25 resting oxygen consumption normalized to lean body mass does not change with aging.26 Therefore, we expect that the load on the diaphragm per cross-sectional area would be similar in young and old rats despite the difference in body weight. In fact, work of breathing per unit body mass is likely elevated in the aging rats due to changes in chest wall compliance.

No effect of MV on diaphragm mass, total protein concentration, or myofibrillar protein concentration was observed, indicating that, unlike 18 h of MV, the 12-h MV protocol used in this study was not sufficient to cause a net loss of diaphragm proteins. Plantaris mass was significantly reduced in the 30-month-old rats compared to the 4-month-old young adults, indicating sarcopenia of locomotor muscles. However, 12 h of MV had no effect on plantaris mass in either age group.

**Costal Diaphragm Contractile Characteristics**

Twitch characteristics on in vitro costal diaphragm strips are presented in Table 2. Maximal twitch

**Table 1—Body Mass, Muscle Mass, and Costal Diaphragm Protein Content**

<table>
<thead>
<tr>
<th>Variables</th>
<th>4 mo Old</th>
<th>12-h MV</th>
<th>30 mo Old</th>
<th>12-h MV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass, g</td>
<td>375.3 ± 16.1</td>
<td>369.4 ± 16.4</td>
<td>529.8 ± 17.4†</td>
<td>517.9 ± 6.9†</td>
</tr>
<tr>
<td>Plantaris mass, mg</td>
<td>368 ± 12</td>
<td>384 ± 12</td>
<td>331 ± 21</td>
<td>337 ± 13</td>
</tr>
<tr>
<td>Plantaris/body mass ratio, mg/g</td>
<td>0.98 ± 0.03</td>
<td>1.04 ± 0.02</td>
<td>0.62 ± 0.03†</td>
<td>0.65 ± 0.02†</td>
</tr>
<tr>
<td>Costal diaphragm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mass, mg</td>
<td>855 ± 43</td>
<td>877 ± 51</td>
<td>1181 ± 34†</td>
<td>1115 ± 63†</td>
</tr>
<tr>
<td>Diaphragm/body mass ratio, mg/g</td>
<td>2.26 ± 0.04</td>
<td>2.37 ± 0.05</td>
<td>2.23 ± 0.04</td>
<td>2.16 ± 0.12</td>
</tr>
<tr>
<td>Total protein, mg/g wet mass</td>
<td>243.81 ± 6.4</td>
<td>226.14 ± 10.2</td>
<td>236.07 ± 3.4</td>
<td>240.09 ± 6.6</td>
</tr>
<tr>
<td>Myofibrillar protein, mg/g wet mass</td>
<td>130.9 ± 5.9</td>
<td>118.5 ± 7.6</td>
<td>125.3 ± 8.4</td>
<td>114.0 ± 9.8</td>
</tr>
</tbody>
</table>

*Values are means ± SEM.
†Significantly different (p < 0.05) from 4-month-old control value.
Specific force was significantly reduced following 12 h of MV in both age groups. However, no age-related difference was observed. The one-half time-to-peak tension (TPT) was significantly longer in the 30-month-old diaphragms, indicating an age-related slowing of contractile speed. One-half TPT was not affected by MV in either age group. No significant group differences were found for rate of tension development, rate of relaxation, or one-half relaxation time.

The mean maximal force-frequency responses for the in vitro diaphragm strips are presented in Figure 1. Note that MV shifts the force-frequency relationship down and to the right in both young and old diaphragms. This cause a similar absolute reduction in force production at all stimulation frequencies. Mean maximal tetanic force was significantly reduced (23%) in the 4-month-old rats by 12 h of MV. Similarly, MV reduced maximal tetanic force (24%) in the 30-month-old rats. Aging is independently associated with a flattening of the force-frequency relationship, especially at higher stimulation frequencies. Mean maximal tetanic force was significantly lower (13%) in the 30-month-old control diaphragms compared to the 4-month-old control diaphragms. Finally, the combination of aging and 12 h of MV (30-month-old, MV) resulted in maximal tetanic forces that averaged 34% lower than the 4-month-old control values. During the fatigue protocol, diaphragmatic force production expressed as a percentage of initial tetanic force did not differ between any of the treatment groups at any of the time points (data not shown).

Diaphragm Glutathione Content

Total glutathione content in the costal diaphragm was significantly lower (36%) in the 4-month-old MV animals compared to the 4-month-old control animals (Fig 2). This is consistent with an acute production of oxidants during the MV period, as is reported during acute exercise, surgical trauma, and hemorrhagic shock. Glutathione content in the 30-month-old control diaphragms was not different from the 4-month-old control diaphragms. There was a trend for mean glutathione content to be lower

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**Table 2—Contractile Twitch Characteristics of In Vitro Costal Diaphragm Strips**

<table>
<thead>
<tr>
<th>Variables</th>
<th>4 mo Old Control</th>
<th>4 mo Old MV</th>
<th>30 mo Old Control</th>
<th>30 mo Old MV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximal twitch force, N/cm²</td>
<td>8.22 ± 0.61</td>
<td>6.18 ± 0.74†</td>
<td>8.58 ± 0.66</td>
<td>7.02 ± 0.56†</td>
</tr>
<tr>
<td>Rate of tension development, g/s</td>
<td>593 ± 65</td>
<td>436 ± 80</td>
<td>583 ± 72</td>
<td>595 ± 68</td>
</tr>
<tr>
<td>Rate of relaxation, g/s</td>
<td>225 ± 23</td>
<td>163 ± 26</td>
<td>226 ± 25</td>
<td>211 ± 21</td>
</tr>
<tr>
<td>½ relaxation time, ms</td>
<td>45.8 ± 5.8</td>
<td>56.0 ± 7.2</td>
<td>54.8 ± 6.4</td>
<td>62.2 ± 6.1</td>
</tr>
<tr>
<td>½ TPT, ms</td>
<td>16.5 ± 0.8</td>
<td>17.0 ± 0.9</td>
<td>19.2 ± 0.9†</td>
<td>19.6 ± 0.8†</td>
</tr>
</tbody>
</table>

*Values are means ± SEM.
†Significantly different (p < 0.05) from 4-month-old control value.
‡Significantly different (p < 0.05) from 30-month-old control value.

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**Figure 1.** Force-frequency relationship for in vitro diaphragm strips from 4-month-old and 30-month-old control and MV rats immediately following 12 h of controlled MV. Values are means ± SEM. *Significantly different (p < 0.05) from 4-month-old control animals. §Significantly different (p < 0.05) from 30-month-old control animals.

**Figure 2.** Total glutathione (GSH) concentration in the costal diaphragm. Values are means ± SEM. *Significantly different (p < 0.05) from 4-month-old control animals.
in the 30-month-old MV diaphragms, compared to 4-month-old control diaphragms, but it did not reach statistical significance (p = 0.11).

Protein Carbonyls

The concentration of protein carbonyls in insoluble protein isolated from the costal diaphragm was significantly elevated (+52%) following MV in both age groups (Fig 3). No age-related differences in total protein carbonyl concentrations were found.

Discussion

It is known that the age of human patients subjected to MV is an independent predictor of ventilator-weaning difficulties. Nonetheless, to our knowledge, this is the first study to directly investigate the effects of age on MV-induced diaphragm contractile dysfunction in animals. It should be noted that the mode of MV used in this study (controlled MV) completely unloads the diaphragm muscle. Clinically, it is much more common for patients to receive MV in a volume- or pressure-assist mode, which requires the diaphragm to initiate the inspiration and trigger the ventilator to assist. Logically, this assist mode of MV would reduce the negative effects of unloading on the diaphragm muscle. Nevertheless, weaning difficulties are common following assist-mode MV. Therefore, we presume that results similar to what we have observed after controlled MV would occur following assist mode of MV, although quantitatively smaller. We chose controlled MV to maximize our ability to detect the MV effects. Future studies should examine the effects of long duration assist mode MV.

Our data reveal that 12 h of controlled MV promotes a similar relative reduction (ie, approximately −24%) in maximal diaphragmatic-specific force generation in young and old animals. Furthermore, compared to nonventilated young adult animals, maximal diaphragmatic-specific force production was significantly (13%) lower in nonventilated senescent rats. Therefore, these findings disclose an additive negative effect of aging and MV on the contractile characteristics of the diaphragm. A brief discussion of these and other major findings follows.

Effects of Aging on the Diaphragm

These data confirm our previous observations of contractile dysfunction in the aging rat diaphragm. Maximal tetanic specific tension was significantly lower (13%) in the diaphragms from 30-month-old control rats, compared to 4-month-old control rats. Twitch characteristics, however, were essentially unaffected by age, with the exception of a significant prolongation in one-half TPT. The mechanism behind the maximal in vitro force deficit is unclear. Unlike the sarcopenia commonly observed in locomotor muscles (eg, 35% reduction in plantaris/body mass ratio in this study), aging is not associated with a loss of diaphragm mass, possibly due to the chronic activity of this muscle. Further, while myofibrillar protein tended to be lower in the 30-month-old diaphragms (mean difference of −4%), this did not reach statistical significance and would not account for the age-related difference in diaphragm-specific force production. Further work will be necessary to determine if the force-generating capacity of individual cross-bridges in the diaphragm is negatively affected by aging.

It has been suggested that oxidative stress is involved in the aging process in skeletal muscle. In fact, we have previously reported that antioxidant enzyme activities are elevated in the aging diaphragm, suggesting increased exposure to radicals during aging. The present study expands and extends our earlier findings by examining markers of cellular oxidation in aging diaphragms. We found no evidence in this study of elevated protein oxidation in the 30-month-old diaphragms compared to the 4-month-old control diaphragms. Furthermore, total glutathione level, which is sensitive to changes in cellular redox status, did not change with age. Therefore, the age-related up-regulation of antioxidant enzymes must be adequate to maintain normal cellular redox status in the diaphragm. These data do not support the idea that age-related changes in diaphragm function are associated with oxidative stress.
Effects of MV on the Aging Diaphragm

Conversely, MV effects on diaphragm function are associated with evidence of oxidative stress. Twelve hours of controlled MV shifts the in vitro force-frequency relationship of costal diaphragm strips down and to the right, causing a reduction in force output at every stimulation frequency > 15 Hz (Fig 1). Twitch characteristics were unaffected by the 12-h MV treatment, suggesting that short-term MV causes deleterious changes in the force-producing elements, rather than in calcium-handling components. Maximal tetanic specific tension was reduced 24%. While there tended to be a reduction in myofibrillar protein concentration with MV (mean difference of –9%), it was not statistically significant and, again, would not seem to explain the force deficit. Perhaps, as has been reported during sepsis, MV causes disassembly of sarcomeric units, which rapidly reduces the force-generating capacity of the muscle. However, myofibrillar protein concentration is not reduced until these disassembled proteins are degraded and removed. This would explain why myofibrillar protein content was not reduced concomitantly with contractile dysfunction after 12 h of MV in this study, while previous experiments examining 18 h of MV did show significant loss of diaphragmatic contractile proteins. Interestingly, there was no interaction between the effects of aging and those of MV. This limits our ability to speculate regarding the mechanisms of age-related effects, but suggest that the mechanisms differ from those that cause MV-related diaphragm dysfunction. Nevertheless, the additive effects of aging and MV caused a dramatic reduction in maximal diaphragmatic strength (34%).

As reported previously for 18 h of treatment, 12 h of controlled MV is associated with an increase in markers of oxidative stress in the costal diaphragm. Our data confirm this in both young adult and aging animals. The 12-h MV treatment caused a significant decrease in glutathione concentration (36%) in the diaphragms of the young rats, and an increase in protein carbonyl concentration (+54%) in the costal diaphragms of both age groups (Fig 3). Since a primary function of glutathione is as a substrate in enzyme-catalyzed elimination of peroxides, it is likely that our MV protocol resulted in an increase in peroxide formation, leading to the oxidation of cellular proteins.

SUMMARY

These novel experiments reveal that 12 h of controlled MV promotes a similar relative reduction (ie, approximately –24%) in maximal diaphragmatic-specific force generation in both young and old animals. Hence, these data do not support the hypothesis that the relative loss of diaphragmatic contractile force following MV will be exacerbated in aged animals. Nonetheless, it is important to consider this MV-induced decrement in diaphragmatic specific tension in the context of the total force decrement of the diaphragm in old animals. That is, compared to nonventilated young adult animals, maximal diaphragmatic specific force production was significantly (13%) lower in control senescent rats. Therefore, compared to healthy young adult animals, the combined aging- and MV-induced decrement in diaphragmatic specific force production was approximately –34% (Fig 1). In other words, despite the similar relative responses of young and old diaphragms to MV, the negative effects of MV are additive to the age-related deficit in diaphragm contractile performance. It follows that this additive effect of aging and MV may explain why patient age is an independent predictor of difficulties in ventilator weaning. Indeed, a reduction in total diaphragmatic-force production in diaphragms from old MV animals would likely place constraints on the diaphragmatic force reserve during periods of increased loads. Therefore, in clinical situations, diaphragms from older patients receiving MV may not be capable of maintaining ventilatory requirements during loading conditions that would occur in patients with obstructive airway diseases.

ACKNOWLEDGMENT: The authors thank Dr. Murat Zergeroglu for technical assistance.

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