Reloading the Diaphragm Following Mechanical Ventilation Does Not Promote Injury*

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Study objective: Mechanical ventilation (MV) is used clinically to treat patients who are incapable of maintaining adequate alveolar ventilation. Prolonged MV is associated with diaphragmatic atrophy and a decrement in maximal specific force production (Po). Collectively, these alterations may predispose the diaphragm to injury on the return to spontaneous breathing (ie, reloading). Therefore, these experiments tested the hypothesis that reloading the diaphragm following MV exacerbates MV-induced diaphragmatic contractile dysfunction, while causing muscle fiber membrane damage and inflammation.

Methods: To test this postulate, Sprague-Dawley rats were randomly assigned to the following groups: (1) control; (2) 24 h of controlled MV; and (3) 24 h of controlled MV followed by 2 h of anesthetized spontaneous breathing. Controls were anesthetized in the short term but were not exposed to MV, whereas MV animals were anesthetized, tracheostomized, and ventilated. Reloaded animals remained under anesthesia, but were removed from MV and returned to spontaneous breathing for 2 h.

Results: Compared to the situation with control animals, MV resulted in a 26% decrement in diaphragmatic specific Po without muscle fiber membrane damage, as measured by an increase in membrane permeability (using the procion orange technique). Further, there were no increases in neutrophil or macrophage influx. Two hours of reloading did not exacerbate MV-induced diaphragmatic contractile dysfunction or cause fiber membrane damage, but increased neutrophil infiltration, myeloperoxidase activity, and muscle edema.

Conclusion: We conclude that the return to spontaneous breathing following 24 h of controlled MV does not exacerbate MV-induced diaphragm contractile dysfunction or result in fiber membrane damage, but increases neutrophil infiltration. (CHEST 2005; 127:2204–2210)

Key words: animal studies; diaphragm; macrophages; weaning

Abbreviations: MPO = myeloperoxidase; MV = mechanical ventilation; MVR = mechanical ventilation with 2 h of reloading; PBS = phosphate-buffered saline; Po = force production

Controlled mechanical ventilation (MV) is used clinically to treat patients who are incapable of maintaining adequate alveolar ventilation. Indications for controlled MV include drug overdose, spinal cord injury, and respiratory failure. After prolonged periods of MV (ie, ≥ 3 days), as many as 20% of patients experience difficulty in weaning from the ventilator.¹ This weaning difficulty may be attributed, in part, to decrements in diaphragmatic contractile function (eg, reduced force production

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In this regard, numerous animal studies have demonstrated that prolonged MV results in diaphragmatic contractile dysfunction. Indeed, as little as 12 h of MV has been reported to decrease the maximal specific Po of the diaphragm by approximately 18%, whereas 48 h of MV has been shown to induce a 60% reduction in diaphragmatic specific Po. Moreover, relatively short periods of MV (ie, 18 h) have been shown to promote proteolysis, atrophy, and oxidative injury to diaphragm muscle fibers. Collectively, these studies indicate that controlled MV results in rapid diaphragmatic atrophy and contractile dysfunction in experimental animals.

MV-induced atrophy and contractile dysfunction in the diaphragm are similar to those observed in locomotor skeletal muscles following prolonged periods of unloading (eg, hindlimb suspension). For instance, significant levels of fiber atrophy and a large (ie, 62%) decline in muscle-specific Po has been reported following prolonged periods of hindlimb suspension. Disuse muscle atrophy in locomotor skeletal muscles has been shown to predispose muscle fibers to injury when the muscle is returned to normal contractile activity. For example, when animals are allowed to reambulate following prolonged periods of muscle unloading, as few as 2 h of reloading the previously inactive muscles results in fiber injury, as revealed both by increases in membrane permeability and by additional decrements in specific Po. Furthermore, this form of muscle fiber injury is associated with inflammatory cell infiltration (ie, neutrophils and macrophages).

To date, it is unknown whether returning to spontaneous breathing following MV results in diaphragmatic injury, inflammation, and an exacerbation of MV-induced contractile dysfunction. Therefore, these experiments determined whether reloading the diaphragm following MV exacerbates contractile dysfunction, and causes membrane damage (ie, increased permeability) and inflammation in the diaphragm. Based on reports of reloading-induced injury and contractile dysfunction in locomotor skeletal muscles, we tested the hypothesis that reloading the diaphragm following 24 h of MV will promote diaphragmatic membrane damage and inflammation along with an exacerbation of contractile dysfunction.

Materials and Methods

Animals and Experimental Design

These experiments were approved by the University of Florida Animal Care and Use Committee and followed the guidelines for animal experiments established by the National Institutes of Health. Healthy, female, Sprague-Dawley rats (4 months old) were randomly assigned to the following groups: (1) control (n = 8); (2) 24 h of MV (n = 6); and (3) 24 h of MV with 2 h of reloading (MVR) [n = 6].

Experimental Protocol

All animals were anesthetized with pentobarbital sodium (60 mg/kg body weight, intraperitoneal). After a surgical plane of anesthesia was achieved, the diaphragms of the control animals were quickly removed, and one segment of the costal diaphragm was used to assess in vitro contractile properties, one strip was incubated in procion orange (see next section for details), and the remaining diaphragm was stored at -80°C for subsequent assay. MV animals were anesthetized, tracheostomized, (using an aseptic technique), and mechanically ventilated (using the control mode) using a volume-driven ventilator (Inspira; Harvard Apparatus; Cambridge, MA). The tidal volume was approximately 0.55 mL per 100 g body weight, with a respiratory rate of 80 breaths/min and a positive end-expiratory pressure of 1 cm H₂O.

The carotid artery was cannulated for the measurement of BP and to allow for blood collection (approximately 80 µL per sample) every 6 h. Blood samples were analyzed for Po₂, PCO₂, and pH using an electronic blood gas analyzer (model 1610; Instrumentation Laboratory; Lexington, MA). Also, the jugular vein was cannulated for the infusion of saline solution and pentobarbital sodium (approximately 10 mg/kg body weight per hour). Animals received an IM injection of glycopyrrolate (0.04 mg/kg) every 2 h to reduce airway secretions. Body temperature was maintained at approximately 37°C, and the heart rate was monitored via a lead II ECG. Continuing care during the MV protocol included lubricating the eyes, emptying the bladder, removing airway mucus, rotating the animal, passive movement of the limbs, and enteral nutrition (Research Diets Inc; New Brunswick, NJ). Finally, note that no neuromuscular blockers were utilized during this study.

After 24 h of MV, animals in the MVR group were removed from the ventilator and were allowed to breathe spontaneously for 2 h while still under anesthesia. Note that the same routine care of animals was undertaken during the reloading period of the experiment. After both specified time points, the diaphragm was removed in the same manner as in the control group. Due to the negative impact of sepsis on the diaphragm, all animals were tested for the presence of Gram-positive and Gram-negative bacteria by blood cultures.

The decision to investigate diaphragmatic injury and contractile dysfunction following the transition from MV to spontaneous breathing was guided by evidence that maximal muscle injury and contractile dysfunction occurs within 2 h following the reloading of previously inactive locomotor muscles. Following 24 h of MV, there is a massive decline in the maximal specific force of the diaphragm (46% decline); these changes could render the diaphragm more susceptible to reloading injury. Furthermore, preliminary experiments in our laboratory have confirmed that MV-induced diaphragmatic contractile dysfunction is maximal at 2 h after the return to spontaneous breathing and is not exacerbated by an additional 6 h of reloading the diaphragm.

Measurement of Diaphragmatic Function and Membrane Damage

The force-frequency response of a strip of costal diaphragm was tested and was normalized to a muscle cross-sectional area, as previously described. Due to technical difficulty, only four animals were included in the MV and MVR groups for contractile function. Furthermore, to assess cellular membrane damage,
costal strip of diaphragm (3 to 4 mm wide) was incubated at an unstrained length for 90 min in oxygenated Krebs solution with procion orange MX2R (Sigma; St. Louis, MO) [0.15% wt/vol]. The sample then was rinsed in Krebs solution, frozen in liquid nitrogen at an unstrained length, and stored at −80°C. Transverse sections (10 μm) were obtained from the procion orange-incubated muscle strip, as described in the previous section. Frozen sections were fixed at room temperature in acetone, dried, and rehydrated in 15 mmol/L phosphate-buffered saline (PBS) solution, pH 7.5. Sections were then blocked for 30 min with 5% normal horse serum. After rinsing in PBS solution, sections were incubated with one of the following primary antibodies for 3 h: (1) anti-ED1; (2) anti-ED2; or (3) anti-W3/13 that binds to an antigen on neutrophils and T cells. T cells are not abundant in this tissue; therefore, almost all of the cells recognized are neutrophils. All antibodies (Serotec; Raleigh, NC) were diluted 1:100, and the primary antibody was replaced with PBS solution for a negative control. Following incubation with the primary antibody, sections were washed in PBS solution and incubated with a biotinylated peroxidases, a solution of 0.3% hydrogen peroxide in methanol was used. After washing in PBS solution for 20 min, sections were incubated (Vectastain Elite ABC kit; Vector Laboratories; Burlingame, CA) for 90 min. To quench endogenous peroxidases, a solution of 0.3% hydrogen peroxide in methanol was used. While blinded, an area of at least 1 mm was sampled, and the number of inflammatory cells (ie, neutrophils, ED1+ macrophages, and ED2+ macrophages) was counted. The volume of the muscle was calculated by multiplying the area by the section thickness (10 μm). Cells were reported as the number of cells per cubic millimeter.

Myeloperoxidase (MPO) activity was determined as described by Seekamp et al. MPO has been shown to correlate with the number of neutrophils present in the tissue. In our laboratory, the coefficient of variation for this assay was <4%. One unit of MPO activity was defined as a change in absorbance of one optical density unit per minute at 480 nm, and activity was expressed as the number of units per gram wet weight.

Finally, the water content of the costal diaphragm was determined by the freeze-drying technique. Samples were placed in a vacuum chamber with a negative pressure of approximately 1 mm Hg. Samples were dried for 24 and 48 h, and were weighed after both time periods. No differences in weight were seen between 24 and 48 h of drying, which ensured that the drying protocol was sufficient.

Assessment of Diaphragmatic Inflammation

Immunohistochemistry was performed to identify neutrophils, ED1+ macrophages, and ED2+ macrophages. Serial sections (10 μm) were obtained from the procion orange-incubated muscle strip, as described in the previous section. Frozen sections were fixed at room temperature in acetone, dried, and rehydrated in 15 mmol/L phosphate-buffered saline (PBS) solution, pH 7.5. Sections were then blocked for 30 min with 5% normal horse serum. After rinsing in PBS solution, sections were incubated with one of the following primary antibodies for 3 h: (1) anti-ED1; (2) anti-ED2; or (3) anti-W3/13 that binds to an antigen on neutrophils and T cells. T cells are not abundant in this tissue; therefore, almost all of the cells recognized are neutrophils. All antibodies (Serotec; Raleigh, NC) were diluted 1:100, and the primary antibody was replaced with PBS solution for a negative control. Following incubation with the primary antibody, sections were washed in PBS solution and incubated with a biotinylated peroxidases, a solution of 0.3% hydrogen peroxide in methanol was used. After washing in PBS solution for 20 min, sections were incubated (Vectastain Elite ABC kit; Vector Laboratories) and developed (NovaRED; Vector Laboratories). Sections were rinsed in water to stop the reaction, dehydrated with ascending alcohols, cleared with Histo-Clear (National Diagnostics; Atlanta, GA), and mounted in permount (Fisher Scientific; Fair Lawn, NJ). Sections were viewed as described in the previous section. While blinded, an area of at least 1 mm was sampled, and the number of inflammatory cells (ie, neutrophils, ED1+ macrophages, and ED2+ macrophages) was counted. The volume of the muscle was calculated by multiplying the area by the section thickness (10 μm). Cells were reported as the number of cells per cubic millimeter.

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Comparisons between groups were made by a one-way analysis of variance and, when appropriate, a Tukey honestly significant difference test was performed post hoc. Significance was established at p < 0.05. Note that our group sample size was selected prior to beginning our experiments using a statistical power analysis. Briefly, based on previous data from our laboratory, four animals per group were required to detect a 10% group difference in the specific force of the diaphragm at an α level of 0.05 and a power of 0.80.

Results

Systemic and Biological Response to MV

Heart rate, systolic BP, arterial pH, PO2, and PCO2 were maintained within a physiologic range during the experimental protocol, as previously reported. Furthermore, there were no significant differences in body weight between groups at the beginning of the experiment, and the MV and MVR protocols did not result in a significant change in body weight. This indicates that our regimen of hydration and nutrition was adequate. All animals tested negative for Gram-positive and Gram-negative bacteria, and had no visual abnormalities of the lungs or peritoneal cavity.

MV-Induced Contractile Dysfunction

Figure 1 illustrates the diaphragmatic force-frequency relationship for all three experimental groups. Compared to the control procedures, MV and MVR resulted in a significant decrease in Po at all stimulation frequencies. Note that no significant differences existed in diaphragmatic Po between MV and MVR (p > 0.05).

Diaphragmatic Histologic Measurements

The incubation of muscle fibers with procion orange is a sensitive histologic technique for assess-
ing membrane damage. That is, procion orange cannot cross intact cell membranes and therefore will enter only muscle fibers with significant membrane disruption. In this regard, no procion orange dye-positive fibers were identified in any of the experimental groups (Fig 2). These results indicate that neither MV nor MVR is associated with significant membrane damage in diaphragmatic fibers.

Immunohistochemical analyses indicated no significant differences in the number of neutrophils, ED1⁺ macrophages, or ED2⁺ macrophages following MV when compared to controls. However, there was a significant increase in the number of neutrophils in the MVR group when compared to that in the MV group (Table 1, Fig 3). In support of these data, no significant differences were noted between the control group and the MV group for MPO activity. However, the MVR group did have significantly increase MPO values when compared to controls (p < 0.05) [Table 1]. Further, MVR resulted in a significant increase in costal diaphragm water content compared to control procedures (p < 0.05) [Table 1]. While there was a trend toward an increase in water content during MV, this difference did not reach significance.

**Discussion**

**Overview of Principle Findings**

Two important and novel findings emerged from these experiments. First, these experiments demonstrated that the contractile dysfunction resulting from 24 h of MV is not due to fiber membrane damage or inflammation in the diaphragm. Second, MVR results in significant neutrophil infiltration and edema without inducing fiber membrane disruption or an exacerbation of MV-induced contractile dysfunction. A brief discussion of these results follows.

**Figure 2.** Cross-section of a diaphragm incubated in procion orange from the following groups: control (top left, A); 24 h of MV (top right, B); 24 h of MVR (bottom left, C); and control diaphragm with crush-induced injury (bottom right, D). This confirms that the procion orange incubation protocol was sufficient to identify membrane damage. Bar = 100 μm.
MV-Induced Contractile Dysfunction Is Not Associated With Increased Membrane Damage or Inflammation

The current investigation confirms previous findings that MV induces diaphragmatic contractile dysfunction (Fig 1).\(^5\)\(^{-10}\) It is noteworthy that studies of locomotor skeletal muscle unloading have also reported contractile dysfunction with muscle disuse.\(^{16,23}\) Nonetheless, the time course of disuse-induced contractile dysfunction differs markedly between the diaphragm and locomotor skeletal muscles. For example, 48 h of MV is sufficient to induce the same diaphragmatic contractile dysfunction as 10 days of disuse in locomotor skeletal muscles using the hindlimb suspension model (ie, Po reduced to approximately 60% below control levels).\(^{5,11,16}\) Furthermore, the 20% loss in muscle mass that occurs during 4 days of hindlimb immobilization\(^{24}\) occurs during only 2 days of MV.\(^7\)

A new and important finding of this study was that the MV-induced decrement in diaphragmatic specific Po is not associated with increased muscle fiber membrane damage. Indeed, our measurements of membrane damage revealed that 24 h of MV does not compromise diaphragm sarcolemma integrity as revealed by the lack of procion orange entry into the cell (Fig 2).

Additionally, our study demonstrates that unloading the diaphragm via controlled MV is not associated with inflammation, as is indicated by the failure to observe an increase in the number of neutrophils, ED1\(^+\) macrophages, or ED2\(^+\) macrophages around diaphragm fibers. This result is in contrast to those of studies\(^{16,17,19}\) reporting a significant increase in inflammatory cells following 10 days of hindlimb muscle unloading. One explanation for this discrepancy is that the duration of MV in our study was not sufficient to induce an inflammatory response. Alternatively, the diaphragm is a unique skeletal muscle and may differ markedly from locomotor skeletal muscles in its response to disuse. For example, the mammalian diaphragm is a long-term active skeletal muscle with a duty cycle ranging from 40 to 45%,\(^5\) whereas hindlimb muscles are intermittently active muscles with duty cycles of 14% and 2%, respectively, for the soleus and extensor digitorum longus muscles.\(^{25}\) These large differences in activity history may play a significant role in the differential responses to disuse between locomotor muscles and the diaphragm. The physiologic differences between the diaphragm and locomotor skeletal muscles have been discussed in detail by Sieck.\(^{25}\)

The current experiments clearly rule out fiber membrane damage and inflammatory injury as the mechanism responsible for MV-induced diaphragmatic contractile dysfunction. Potential mechanisms that would explain the MV-induced diaphragmatic weakness are numerous, and include impaired excitation-contraction coupling, proteolytic degradation of essential contractile or cytoskeletal proteins, oxidative modification of key contractile proteins, and reduced levels of contractile proteins within the sarcomere.\(^{11}\) At present, the mechanism responsible for MV-induced diaphragmatic weakness is unknown and remains an important area for future research.

Reloading the Diaphragm Following MV Does Not Exacerbate Diaphragm Dysfunction or Membrane Damage

A novel and important finding in the present study was that reloading of the diaphragm following 24 h of MV does not result in diaphragm membrane damage (ie, increased permeability) and that reloading does not exacerbate the MV-induced contractile deficit in the diaphragm. In contrast to these findings, reloading the locomotor skeletal muscle after 10 days of hindlimb suspension results in muscle injury, as evidenced by an additional 50% reduction in muscle-specific Po.\(^{16}\) Similarly, Nuygen and Tidball\(^{20}\) have reported that while prolonged unloading of murine skeletal muscle does not result in an increase in fiber membrane damage, the reambulation of these animals is associated with significant muscle membrane injury. At least two plausible explanations exist for these divergent findings. First, it is possible that the relative muscle stress associated with reloading the

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control Group (n = 8)</th>
<th>24 h MV Group (n = 6)</th>
<th>MVR Group (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils, cells/mm(^3)</td>
<td>1.368 ± 173</td>
<td>937 ± 164</td>
<td>1.892 ± 376†</td>
</tr>
<tr>
<td>ED1, cells/mm(^3)</td>
<td>2.808 ± 507</td>
<td>3.527 ± 816</td>
<td>5.000 ± 683</td>
</tr>
<tr>
<td>ED2, cells/mm(^3)</td>
<td>15.144 ± 1.237</td>
<td>12.088 ± 1.391</td>
<td>14.018 ± 1.461</td>
</tr>
<tr>
<td>MPO, U/gww</td>
<td>1.31 ± 0.025</td>
<td>1.35 ± 0.027</td>
<td>1.58 ± 0.038‡</td>
</tr>
<tr>
<td>Water content, %</td>
<td>75.82 ± 0.75</td>
<td>78.19 ± 0.92</td>
<td>79.64 ± 0.35‡</td>
</tr>
</tbody>
</table>

*Values given as mean ± SEM. ED1 = ED1\(^+\) macrophages; ED2 = ED2\(^+\) macrophages; U/gww = units per gram wet weight.
†Significant difference vs control group.
‡Significant difference vs 24 h of MV group.
soleus by ambulation differs from the load of breathing imposed on the diaphragm. That is, the load encumbered by the diaphragm during normal breathing is potentially less than the load burdened on the hindlimb during normal ambulation. Therefore, in contrast to the soleus, the load imposed on the diaphragm by the return to spontaneous breathing may not reach the load threshold that is required to induce muscle injury.

A second possible explanation is that 24 h of MV is insufficient to produce the level of diaphragmatic atrophy required to increase the risk of reloading injury. For example, 18 h of MV results in a 7% decrease in costal diaphragm mass, whereas 10 days of hindlimb unloading decreases soleus muscle mass by 42%. Therefore, it is possible that longer periods of MV, resulting in more extensive diaphragmatic atrophy, could increase the risk of reloading injury in the diaphragm.

A final point of interest is that, compared to MV alone, the return to spontaneous breathing following MV resulted in a small but significant increase in the number of neutrophils and MPO activation within the diaphragm (Table 1). Nonetheless, the observation that reloading the diaphragm following MV did not exacerbate MV-induced contractile dysfunction or promote diaphragm membrane injury indicates that the observed level of neutrophil invasion in the reloaded diaphragm does not induce diaphragmatic injury or contractile dysfunction.

Critique of Experimental Model

Due to the invasive nature of obtaining a diaphragm sample, an animal model must be utilized for studying the effects of MV on the diaphragm. The reasons for using the rat as our experimental model were twofold, as follows: (1) the rat is of adequate size for conducting the necessary surgical techniques and also allows for the removal of several blood samples for monitoring blood gas homeostasis; and, most importantly, (2) the rat and human diaphragms have very similar anatomic and functional features as well as a similar fiber type composition.

For the current investigation, we chose controlled MV vs pressure-assist MV. Controlled MV results in the complete inactivation of the diaphragm and is clinically used in numerous situations including drug overdose, spinal cord injury, and surgery, and is commonly used in pediatric patients.

Finally, pentobarbital sodium was chosen as the anesthetic. This barbiturate has no effect on the function of the diaphragm as was previously shown by anesthetizing animals and allowing them to spontaneously breathe without the aid of MV. Therefore, spontaneously breathing animals were not included in this investigation.

Summary and Conclusions

For the first time, these experiments have revealed that MV-induced diaphragmatic contractile dysfunction is not due to increased fiber membrane damage or inflammation. Furthermore, the return to spontaneous breathing after 24 h of MV is not associated with diaphragm membrane damage or additional contractile dysfunction. This finding may have clinical relevance in regard to weaning patients from the ventilator following short periods of controlled MV. However, the time course in which the diaphragm recovers from MV is unknown. Future
experiments investigating longer durations of MV followed by various reloading periods are required to determine whether days to weeks of MV increases the risk of diaphragmatic injury during attempts to wean patients from the ventilator by the return to spontaneous breathing.

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