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Rapid Onset of Specific Diaphragm Weakness in a Healthy Murine Model of Ventilator-induced Diaphragmatic Dysfunction

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ABSTRACT

Background: Controlled mechanical ventilation is associated with ventilator-induced diaphragmatic dysfunction, which impedes weaning from mechanical ventilation. To

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What We Already Know about This Topic

- Ventilator-induced diaphragm dysfunction has been demonstrated in various animal models but not mouse

What This Article Tells Us That Is New

- Six hours of mechanical ventilation in mice reduced the diaphragm contraction force by 40% without muscle atrophy or sarcolemmal injury
- The first mouse model of ventilator-induced diaphragm dysfunction is presented

design future clinical trials in humans, a better understanding of the molecular mechanisms using knockout models, which exist only in the mouse, is needed. The aims of this study were to ascertain the feasibility of developing a murine model of ventilator-induced diaphragmatic dysfunction and to determine whether atrophy, sarcolemmal injury, and the main proteolysis systems are activated under these conditions.

Methods: Healthy adult male C57/BL6 mice were assigned to three groups: (1) mechanical ventilation with end-expiratory positive pressure of 2–4 cm H₂O for 6 h (n = 6), (2) spontaneous breathing with continuous positive airway pressure of 2–4 cm H₂O for 6 h (n = 6), and (3) controls with no specific intervention (n = 6). Airway pressure and hemodynamic parameters were monitored. Upon euthanasia, arterial blood gases and isometric contractile properties of the diaphragm and extensor digitorum longus were evaluated. Histology and immunoblotting for the main proteolysis pathways were performed.

Results: Hemodynamic parameters and arterial blood gases were comparable between groups and within normal physiologic ranges. Diaphragmatic but not extensor digitorum longus force production declined in the mechanical ventilation group (maximal force decreased by approximately 40%) compared with the control and continuous positive airway pressure groups. No histologic difference was found between groups. In opposition with the calpains, caspase 3 was activated in the mechanical ventilation group.

Conclusion: Controlled mechanical ventilation for 6 h in the mouse is associated with significant diaphragmatic but not limb muscle weakness without atrophy or sarcolemmal injury and activates proteolysis.

MECHANICAL ventilation (MV) is a life-saving procedure in many critically ill patients, but a short period of MV has been associated with ventilator-induced lung injury even in healthy lungs.¹ In addition, controlled MV, which maintains the diaphragm at rest, has been implicated in the development of diaphragmatic dysfunction not only in animal models^{2–7} but also in humans.^{8–12} This entity, which has been termed ventilator-induced diaphragmatic dysfunction (VIDD),¹³ is likely to play an important role in the difficulties frequently encountered in weaning critically ill patients from the ventilator.¹⁴

Multiple mechanisms appear to be involved in VIDD, such as an increase of reactive oxygen species, mitochondrial dysfunction, inhibition of the insulin-like growth factor pathway, and activation of different proteolytic systems, such as the calpains and caspase 3. Calpains and caspase 3 are ubiquitous nonlysosomal proteases, and their expression may be up-regulated early in the time course of VIDD.^{10,15,16} All of these mechanisms culminate in muscle atrophy,^{17–19} injury,¹⁰ and a loss of diaphragmatic force-generating capacity.

Although the precise time course for development of VIDD is unknown in humans, two studies reported that diaphragmatic force is impaired after 24 h of MV.^{8,10} In animal models, VIDD has been described after controlled MV of 12 h in rats,²⁰ 24 h in rabbits,⁶ and 48 h in piglets.⁴ Maintaining stable vital signs and gas exchange in mechanically ventilated experimental animals for such prolonged periods of time is difficult and resource intensive, and the impact of a shorter period of time on diaphragm properties has not been described. In addition, although antioxidant therapies and proteolysis inhibitors have been reported to mitigate VIDD when administered before MV initiation in animal models,^{21–23} research into the specific pathophysiologic mechanisms underlying VIDD has been hampered by the lack of a corresponding murine model, which would permit a greater application of modern molecular biology and genetics tools to the problem.

Therefore, the aims of the current study were first to assess the feasibility and validation of VIDD in the mouse after 6 h of MV and second to explore both histologic and the main protease pathways after 6 h of MV.

Materials and Methods

The study followed the guidelines for animal experiments established by the institutional animal care committee (INSERM U 1046, Montpellier, France) and the recommendations of the Helsinki Declaration. To estimate the duration of MV needed to induce VIDD in the mouse, we first referred to published studies reporting the earliest time point of VIDD occurrence in other animal models,^{4,6,20} which we defined as a statistically significant drop in diaphragm-specific force generation. We then plotted the value of protein turnover rate data in different animal species^{24,25} and the reported time points for the onset of VIDD and hypothesized that 6 h of MV would induce VIDD in mice.

Healthy male (10–12 weeks, 25–30 g) C57/BL6 mice were maintained in our animal facility for 1 week before initiation of the experiments. Mice were then randomly assigned to one of three experimental groups: (1) MV for 6 h (MV group, $n = 6$), (2) continuous positive airway pressure for 6 h (CPAP group, $n = 6$), and (3) controls (CTRL group, $n = 6$), which were euthanized by intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight) and exsanguination without any previous intervention.

Experimental Protocol for MV and CPAP Groups

Mice in the MV and CPAP groups were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight) and orally intubated with a 22-gauge angiocatheter. No neuromuscular blocking agents were used. To control for the potential effects of anesthesia and intubation over a 6-h period, CPAP mice were treated in a manner identical to that of MV group but breathed spontaneously throughout the experiment. CPAP 3–4 cm H₂O was achieved through the use of an air compressor to deliver a high inspiratory flow rate (1 l/min room air) while the expiratory port was placed under a water seal. General care of the MV and CPAP groups included continuous maintenance of body temperature using a homeothermic blanket (Homeothermic Blanket Control unit; Harvard Apparatus, Saint-Laurent, Quebec, Canada) and hourly intraperitoneal injections of 0.05 ml Ringer's lactate solution to maintain hemodynamic stability and compensate insensible losses. Bladder expression, ocular lubrication, and passive limb movements also were performed.

Mechanical ventilation was performed with a small animal ventilator (Minivent®, Harvard Apparatus) using the following ventilator settings: fraction of inspired oxygen 0.21 (room air), controlled volume mode with tidal volume 10 μ l/mg body weight, respiratory rate 150 breaths/min, and positive end-expiratory pressure 3–4 cm H₂O achieved by placing the expiratory port under a water seal. MV was set to have minute ventilation slightly below the apneic threshold. Peak airway pressure and positive end-expiratory pressure were monitored at the airway opening using a calibrated pressure transducer (CD 15 Carrier Demodulator; Validyne®, Northridge, CA), and these data were acquired *via* a

computerized data acquisition system (Acqknowledge®, version 3.5.3; BIOPAC Systems Inc., Goleta, CA). Clinical examination and airway pressure curve analysis were used to ensure a lack of spontaneous respiratory efforts during MV. After intubation, a Millar® catheter transducer (MIKRO-TIP®, model PVR-10H5; Millar Instrument, Houston, TX) was placed in the carotid artery for blood pressure and heart rate monitoring. These data were recorded *via* a computerized data acquisition system (IOX2, version 2.4.1.10; EMKA Technologies, Paris, France). Blood was collected from the carotid artery at the end of the experiment, and gas exchange was assessed using a blood gas analyzer (Roche OMNI, Mannheim, Germany).

Measurement of Diaphragm and Extensor Digitorum Longus (EDL) Contractile Properties

At the end of the experiment in the three groups, mice were euthanized by exsanguination, and the diaphragm and EDL were surgically removed to permit measurements of isometric contractile properties as described in detail previously.²⁶ The muscles were supramaximally stimulated using square wave pulses (Model S48; Grass Instruments, West Warwick, RI).^{27,28} The force–frequency relationship was determined by sequentially stimulating the muscles for 600 ms at 10, 20, 30, 50, 60, 80, 100, and 120 Hz with 1 min between each stimulation train. Fatigability of the muscles was assessed by measuring the loss of force in response to repeated stimuli (30 Hz, 300 ms duration) over 10 min for the diaphragm and 5 min for the EDL. After the measurements of contractile properties were completed, muscles were measured at Lo (the length at which the muscle produced maximal isometric tension), dried quickly to remove the buffer, and weighed. The muscle cross-sectional area was determined by dividing muscle weight by its length and tissue density (1.056 g/cm³). Diaphragmatic and EDL force production were then normalized to the muscle cross-sectional area to determine the specific force, which is expressed in newtons per square centimeter.

Measurement of Atrophy and Diaphragm Lesions (Histology)

Atrophy. Ten-micrometer unfixed cryostat sections of the diaphragms from the three groups were stained by hematoxylin and eosin and visualized under a Nikon optiphot-2 microscope (Nikon Instruments, B.V. Amstelveen, The Netherlands). All diaphragm images were obtained under identical conditions and with the same objective lens. The shape of each muscle fiber was accurately defined (on images \times 400), and a schematic drawing of each stained diaphragm section was recorded. Schematic representations of each muscle section were analyzed by the Histolab program (version 5-13-1; Microvision Instrument, Evry, France). On each schematic drawing, the fiber cross-sectional areas were measured as a surrogate of diaphragm atrophy.²⁷

Lesion. The level of acute sarcolemmal injury in diaphragm myofibers was evaluated by exposing muscles to a fluorescent

tracer dye (Procion orange; Sigma, St. Louis, MO). Because the sarcolemma of normal myofibers is impermeable to Procion orange, fibers with sarcolemmal damage can be identified by their inability to exclude Procion orange from the cytoplasm.²⁸ A muscle strip from each diaphragm was immersed for 60 min in Procion orange solution and continuously oxygenated at room temperature. The muscles subsequently were rinsed, snap-frozen in isopentane precooled with liquid nitrogen, and stored at -80°C . Frozen sections were mounted for viewing under epifluorescence microscopy, and the images were captured to computer using a video camera. The number of myofibers demonstrating clear cytoplasmic staining was determined. A minimum of 200 myofibers from randomly selected microscopic fields was counted on each tissue section.

Biochemical Evaluation

Immunoblot analysis was performed to evaluate the ratios of the active (cleaved) forms of calpains 1 and 2 to total levels of the two calpain isoforms. The total and cleaved form of caspase 3 also were measured.

Blots were incubated with specific antibodies for calpain 1 (clone 15C10 1:1,000 dilution, Sigma), calpain 2 (ab39165 1:1000 dilution; Abcam, Cambridge, MA), and for primary anticaspase 3 (1:500) at 4°C overnight (Cell Signaling, Danvers, MA). The membrane was then incubated with secondary antibody (antirabbit 800 nm; 1:30,000) for 1 h in the dark. After the final washes, the membrane was scanned using Odyssey™ Infrared Imager (LI-COR® Biosciences, Lincoln, NE).

Statistical Analysis

Data are presented as mean \pm SE unless specifically indicated. Statistical significance was assessed using the Student *t* test. Hemodynamic, blood gas, and muscle contractility force–frequency responses were analyzed using the Kruskal-Wallis test taking into account the return to the baseline between each force measure. Differences in muscle contractility for fatigability between groups were compared using a mixed regression model taking into account both time (as repeated measures were performed and analyzed) and group effect in the comparison model. Group and time were considered as fixed, and individual animals were considered a random effect. No specified covariance was used. Statistical analysis was performed using Statview, version 5.0 (SAS, Cary, NC) by an independent statistician (N.M.). Significance was established at $P < 0.05$.

Results

General Characteristics

Mean body weight values did not differ significantly between the MV, CPAP, and CTRL groups (28.4 ± 1.9 , 27.2 ± 1.9 , and 26.9 ± 1.4 g, respectively). For the MV group, additional sodium pentobarbital injection was given when spontaneous

Table 1. Arterial Blood Gases Obtained in Controlled Mechanical Ventilation and Continuous Positive Airway Pressure Groups at the End of the Experiment

Group	pH	PaO ₂ (mmHg)	Paco ₂ (mmHg)	HCO ₃ ⁻ (mM)
MV (n = 6)	7.40 ± 0.02	70.8 ± 6.7	36.1 ± 2.5	21.9 ± 0.9*
CPAP (n = 6)	7.40 ± 0.03	80.9 ± 8.8	44.0 ± 4.1	26.4 ± 1.3

Data are represented as mean ± SE.

* $P < 0.05$ between the MV and CPAP groups.

CPAP = continuous positive airway pressure; MV = controlled mechanical ventilation.

breathing efforts were detected either clinically or on the airway pressure curve. However, the total amount of pentobarbital administered during the experiments was not significantly different between the MV and CPAP groups (0.12 ± 0.01 mg/g and 0.11 ± 0.01 mg/g body weight, respectively).

Respiratory and Hemodynamic Monitoring

During the 6-h period of MV, mean peak airway pressure was 14.0 ± 1.3 cm H₂O, and mean positive end-expiratory pressure was 3.3 ± 0.2 cm H₂O. In the CPAP group, the mean positive end-expiratory pressure was 4.0 ± 0.9 cm H₂O ($P = 0.17$ vs. MV group). Table 1 shows arterial pH, PaO₂, Paco₂, and bicarbonate (HCO₃⁻) values after 6 h of MV or CPAP. Both hemodynamic parameters and arterial blood gas measurements were stable and within the physiologic range throughout the duration of the study (table 2), although bicarbonatemia was higher in the CPAP group than the MV group.

Isometric Contractile Properties of the Diaphragm

Compared with CTRL and CPAP groups, 6 h of MV resulted in a significant decrease of diaphragmatic force production at all stimulation frequencies ($P = 0.01$) (fig. 1). However, no significant difference in diaphragmatic force production was observed between the CTRL and CPAP groups. For example, at 120 Hz diaphragmatic force was, respectively, 27.4 ± 3.3 , 27.3 ± 8.8 , and 15.9 ± 3.2 N/cm² for the CTRL, CPAP, and MV groups ($P < 0.05$ between MV and both CTRL and CPAP groups). In addition, during the imposition of repetitive trains of electrical stimulation to induce muscle fatigue, diaphragm-specific force in the MV group remained significantly lower than in the CTRL and CPAP groups (fig. 2).

Isometric Contractile Properties of Limb Muscle

In contrast to the diaphragm, contractile properties of the EDL did not differ among the MV, CPAP, and CTRL groups. This was true with respect to both the force–fre-

quency relationship and the response to fatiguing muscle contractions (figs. 3 and 4).

Histologic Findings

Six hours of MV was not associated with atrophy or sarcolemmal lesion (fig. 5).

Biochemical Evaluation

There were no significant differences among the three experimental groups in the concentrations of cleaved protein for either of the two calpain isoforms (fig. 6). Interestingly, cleaved caspase 3 was increased in the MV group compared with the CTRL and the CPAP groups (fig. 7).

Discussion

The current study is the first to demonstrate the development of VIDD in mice. The results of our investigation confirmed our hypothesis that a 6-h period of MV would result in a significant reduction of diaphragmatic specific force in mice (mean decrease in maximal force of approximately 40%), without any significant impairment of limb muscle contractile properties. Importantly, the existence of a mouse model of VIDD opens opportunities for performing more detailed mechanistic studies than have been possible to date. Thus, the application of this model to transgenic or knockout mice will enable the examination of specific molecular pathways implicated in the pathophysiology of VIDD and will help in finding specific strategies to prevent and/or limit VIDD in humans.¹⁸

Before discussing the implications of our findings in more detail, potential confounding factors and certain technical aspects of the study are addressed. First, to ensure that our MV protocol was successful in maintaining hemodynamic stability, we demonstrated that both hemodynamic parameters and arterial blood gas measurements were stable and within the physiologic range throughout the duration of the

Table 2. Hemodynamic Parameters Obtained in Controlled Mechanical Ventilation Group (n = 6) during the Experiment

	Hour 1	Hour 2	Hour 3	Hour 4	Hour 5	Hour 6
Systolic blood pressure (mmHg)	140 ± 10	141 ± 9	137 ± 11	138 ± 8	148 ± 8	153 ± 6
Heart rate (beats/min)	477 ± 28	470 ± 39	481 ± 43	474 ± 39	484 ± 42	507 ± 35

Data are represented as mean ± SE. No significant differences were observed across time points.

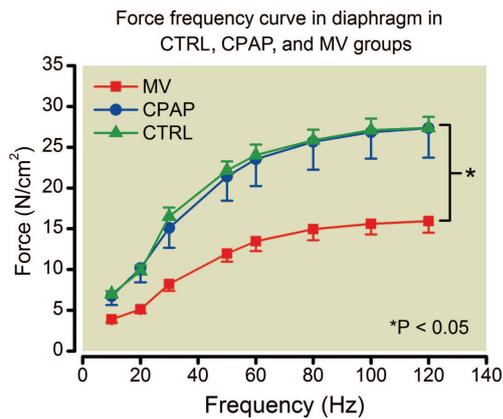


Fig. 1. Force–frequency curves obtained in diaphragm after 6 h of mechanical ventilation (MV) and/or continuous positive airway pressure (CPAP) and in the control (CTRL) group. Values are means \pm SE. * $P < 0.05$ for group main effect of MV versus CTRL and CPAP groups.

study. Therefore, tissue hypoperfusion cannot account for the reductions of diaphragmatic force production observed in the MV group.²⁹ Second, to achieve an absence of spontaneous respiratory efforts, mice in the MV group were maintained at a level of ventilation intended to be slightly below the apneic threshold.³⁰ As a result, the PaCO_2 in the MV group showed a nonsignificant trend toward lower values compared with that in the CPAP mice, along with a small compensatory reduction in bicarbonate to maintain a normal pH (table 1). Physiologic values of PaCO_2 in mice have been reported to be as low as 33 mmHg.³¹ In addition, because severe hypercapnia, rather than hypocapnia, is associated with diaphragmatic fatigue and weakness, the small variations in PaCO_2 observed in our study cannot plausibly explain the large reductions of diaphragmatic force production found in the MV group.³² Third, despite careful airway pressure curve monitoring to detect any spontaneous inspiratory effort, some nondetectable diaphragmatic contractions may

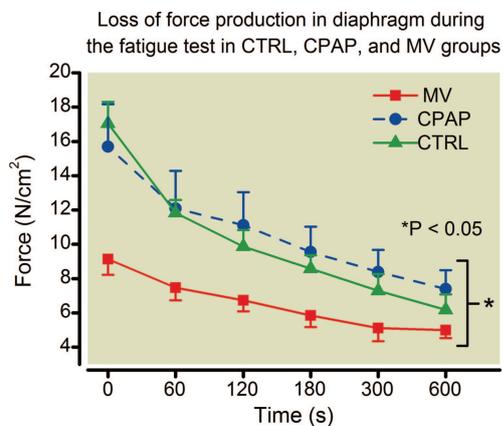


Fig. 2. Comparison of the loss of absolute force production of the diaphragm during the fatigue test after 6 h of mechanical ventilation (MV) and/or continuous positive airway pressure (CPAP) and in the control (CTRL) group. Values are means \pm SE. * $P < 0.05$ for group main effect of MV versus CTRL and CPAP groups.

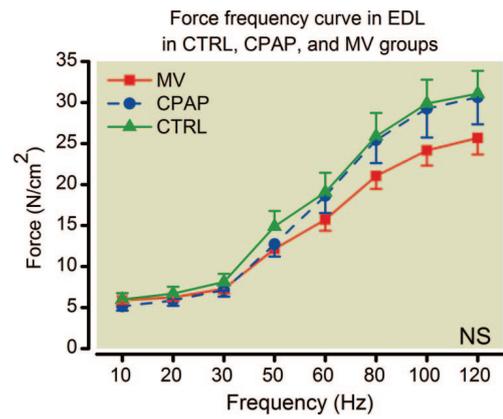


Fig. 3. Force–frequency curves obtained in extensor digitorum longus (EDL) after 6 h of mechanical ventilation (MV) and/or continuous positive airway pressure (CPAP) and in the control (CTRL) group. Values are means \pm SE. No significant differences were detected. NS = not significant.

have occurred. Finally, small numbers of animal per group could, in theory, impair the power of the study. However, we did not observe any tendency in the biochemical or histologic parameters that could have any scientific relevance.

To isolate potential confounding factors, we included a spontaneously breathing intubated group treated with CPAP. The dose of pentobarbital was not significantly different between the CPAP and MV groups. Because muscle contractility parameters in the CPAP group did not differ from those of the CTRL group, which was not subjected to any of these interventions, we conclude that neither positive end-expiratory pressure nor prolonged anesthesia had significant effects upon diaphragmatic function in our study.

In the current study, diaphragmatic force impairment was not associated with sarcolemmal injury. Studies have reported sarcolemmal injury during MV starting after 50 h in rabbits,³³ 5 days in pigs,³⁴ and 2–5 days in humans,¹⁰ and it is likely that a 6-h period is too short to observe sarcolemmal

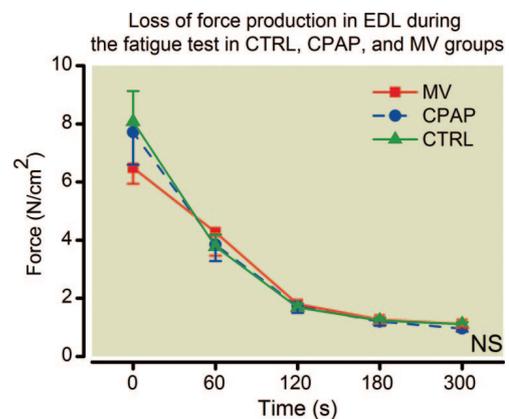


Fig. 4. Comparison of the loss of absolute force production in extensor digitorum longus (EDL) during the fatigue test after 6 h of mechanical ventilation (MV) and/or continuous positive airway pressure (CPAP) and in the control (CTRL) group. Values are means \pm SE. No significant differences were detected. NS = not significant.

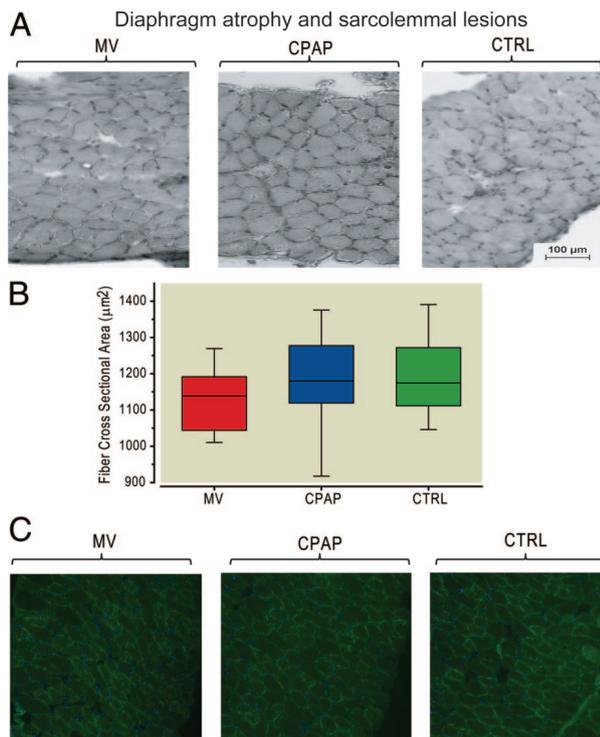


Fig. 5. Comparison of representative mechanical ventilation (MV) and/or continuous positive airway pressure (CPAP) and in the control (CTRL) group diaphragm-biopsy specimen with respect to fiber size (A). Schematic representation (on images $\times 400$) of each muscle section was analyzed, and data were averaged per approximately 500 muscle fibers from each dissected diaphragm of the control, CPAP, and MV groups. There was not any difference between groups (B). Evaluation of acute sarcolemmal injury induced by 6 h MV in mice. Intracellular Procion orange dye uptake by diaphragmatic myofibers indicates the presence of sarcolemmal injury. There was not any diaphragm sarcolemmal injury in the MV, CPAP, and CTRL groups (C). Values are median and twenty-fifth to seventy-fifth quartiles.

injury. We also showed that atrophy was not present after 6 h of MV in healthy mice (fig. 5), as was reported in a study performed in rats.³⁵ In addition, although atrophy has been described in animal and human studies,^{7,10,12,27} studies have shown that the force loss is persistent even after correcting for any reduction in muscle cross-sectional area.^{6,7,20} Atrophy and decreased diaphragmatic force can be uncoupled from one another. For example, preventive treatment with apocynin (a calpastatin up-regulator that decreases calpain activity and thus limits proteolysis) limited diaphragm force impairment in MV rats but failed to prevent atrophy. The uncoupling between atrophy can be explained by the disassembly of actomyosin complexes by proteolytic enzymes (calpains, caspase) but also by others mechanisms that have been reported in other fields, such as calcium homeostasis³⁶ or titin degradation, a protein essential to maintaining sarcomere structural and mechanical stability during calcium activation.³⁷ Our results suggest that even a few hours of controlled MV may be detrimental for diaphragmatic force, even

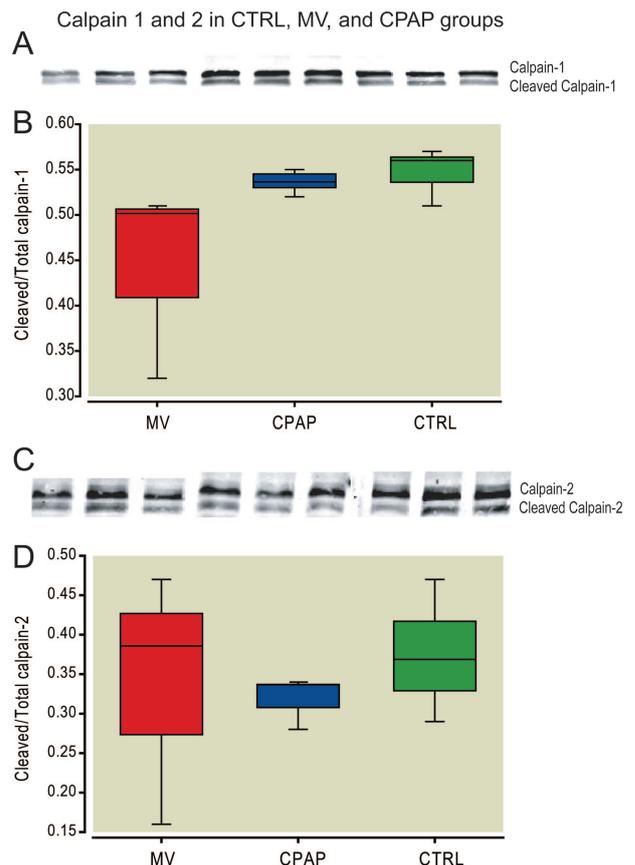
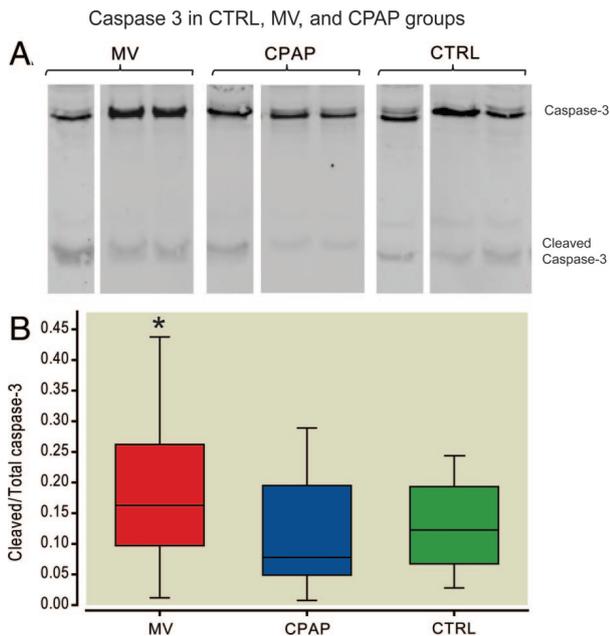


Fig. 6. Expression of calpain isoforms in diaphragms of the mechanical ventilation (MV), continuous positive airway pressure (CPAP), and control (CTRL) groups. Representative immunoblots (A and C) and group mean quantification of protein concentrations measured in diaphragm tissues obtained from the MV, CPAP, and CTRL groups for calpain 1 (B) and calpain 2 (D). Values are median and twenty-fifth to seventy-fifth quartiles. NS = not significant.

in the absence of lesion or atrophy. However, the impact of a short period (less than 24 h) of controlled MV on diaphragmatic force in humans and its potential consequences on weaning are unknown.

To explore the mechanisms that might have led to the early onset of VIDD in the murine model, we measured the quantities of total and cleaved calpains 1 and 2 and caspase 3 in the diaphragm. The calpains are cysteine proteases involved in multiple functions, including regulation of the cell cycle, apoptosis, and cytoskeletal reorganization. In addition, calpains play a role in muscle wasting by initiating myofibrillar disassembly, which makes the myofibrillar proteins amenable to further degradation by the proteasome.^{15,38} Previous investigations have reported calpain activation after longer periods of MV (12–24 h) in rats. Interestingly, calpains 1 and 2 did not appear to play a role in our findings, because concentrations of the active cleaved forms of these proteins were not altered in the mechanically ventilated diaphragms after 6 h, despite the major reduction in specific force observed at the same time point. In contradistinction to the calpains, we observed a higher caspase 3 activity in the



MV group compared with the CPAP group (fig. 7). Numerous cellular mechanisms lead to an early caspase 3 activation,³⁹ which might explain the earlier activation than that of the calpains, including mitochondrial-dependent (cytochrome c release) and mitochondrial-independent (*e.g.*, calcium activation of calpain) pathways.³⁹ Caspase activation results in protein cleavage, stimulates apoptosis, and has been implicated in atrophy in both animal³⁵ and humans.¹² In the current study, we report that 6 h of MV activates caspase 3 in mice and thus may participate in the early diaphragm decrease of contractility. Interestingly, interventional studies have started to evaluate caspase inhibitor in humans,⁴⁰ and it may be of interest to evaluate those inhibitors in the current field in humans. Other mechanisms implicated in VIDD, such as oxidative stress possibly caused by impaired mitochondrial function,⁴¹ are likely to underlie the earliest phases of diaphragmatic weakness associated with MV.

Finally, the results of this investigation support our hypothesis that the rapidity of VIDD onset may be a function of the protein turnover rate that is present before the initiation of MV. As shown in figure 8, in comparing different animal species one finds an inverse (and possibly exponential) relationship between basal protein turnover rate and the earliest time at which VIDD has been documented. It should be noted that these data are based on studies performed in previously healthy animals.^{24,25} In critically ill patients, the whole body protein turnover rate often is substantially increased.⁴² Therefore, we propose that in such patients the

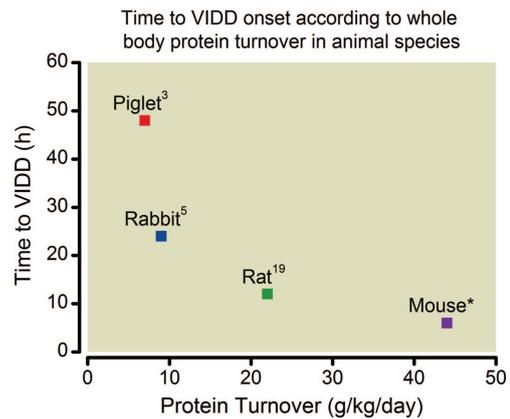


Fig. 8. Time to ventilator-induced diaphragmatic dysfunction (VIDD) as a function of whole body protein turnover rate in different animal models.^{23,24} A more rapid onset of VIDD is associated with a higher basal protein turnover rates. *The current study.

time to onset of VIDD after the initiation of MV is likely to be greatly accelerated.

Conclusion

We report for the first time that 6 h of controlled MV leads to a severe impairment of diaphragmatic contractility in mice without a significant change in limb muscle function. Six hours of controlled MV was associated with an increase of caspase 3 activity but not with any change in calpain 1 or 2 activities, atrophy, or lesion. The existence of this murine model will permit investigations using genetically modified mice, which will advance our understanding of VIDD pathophysiology and the development of specific treatments in humans.

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