Altered diaphragm contractile properties with controlled mechanical ventilation

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Sassoon, Catherine S. H., Vincent J. Caiozzo, Albana Manka, and Gary C. Sieck. Altered diaphragm contractile properties with controlled mechanical ventilation. J Appl Physiol 92: 2585–2595, 2002.—This study shows that, over time, diaphragm inactivity with controlled mechanical ventilation (CMV) decreases diaphragm force and produces myofibril damage contributing to the reduced force. We measured in vivo and in vitro diaphragm contractile and morphological properties in 30 sedated rabbits grouped (n = 6) as follows: 1 or 3 days of CMV, 1 or 3 days of 0 cmH2O continuous positive airway pressure, and control. The CMV rate was set sufficient to suppress diaphragm electrical activity. Compared with the control group, phrenic-stimulated maximum transdiaphragmatic pressure did not decrease with continuous positive airway pressure but decreased to 63% after 1 day of CMV and to 49% after 3 days of CMV. The in vitro tetanic force decreased to 86% after 1 day of CMV and to 44% after 3 days of CMV. After 3 days of CMV, significant myofibril damage occurred in the diaphragm but not in the soleus. The decrease in tetanic force correlated with the volume density of abnormal myofibrils. We conclude that CMV had a detrimental effect on diaphragm contractile properties.

passive ventilation; diaphragm inactivity; diaphragm muscle force; force-frequency relationship; myosin heavy chain isoform expression

ACUTE RESPIRATORY FAILURE is the most frequent indication for the application of mechanical ventilation (8). In patients with acute respiratory distress syndrome, the application of volume- or pressure-controlled mechanical ventilation may require prolonged heavy sedation with or without neuromuscular blockade (29). Under those conditions, the ventilator assumes the patients’ entire work of breathing, or patient ventilation is passive. In two animal studies (1, 21), passive ventilation appears to induce detrimental effects on diaphragm muscle function. In sedated and paralyzed baboons, transdiaphragmatic pressure (Pdi) and endurance decreased significantly after 11 days of controlled mechanical ventilation (CMV) (1). In anesthetized rats, 2 days of CMV reduced diaphragm muscle force-generating capacity by 42% compared with control animals breathing spontaneously (21). Unfortunately, in both studies, the control group did not receive the same duration of anesthesia, sedation, or paralyzing drugs. The confounding effects of paralyzing agents (26) with or without anesthetic drugs (33) may have played a role in the reduced diaphragm muscle function. In addition, neither study assessed the time course of the decrease in diaphragm muscle force.

The cause of the decrease in diaphragm muscle force is unclear and is likely multifactorial. This may involve the excitation-contraction coupling apparatus with (18, 42) or without the contractile machinery. Reduced myofibril protein (13) or structural changes may have a detrimental effect on the contractile machinery. Indeed, ultrastructural changes in the form of myofibril disruption and mitochondrial swelling have been demonstrated in immobilized hindlimb skeletal muscle of rabbits (20). As with skeletal muscle, when the diaphragm is exposed to inactivity with phrenic denervation, it undergoes functional and structural changes in the form of myofibril disruption and Z-band streaming (15, 41). The diaphragm is also inactive during CMV, but there is limited information on the structural changes of the diaphragm. These structural changes, if any, may contribute to the decrease in diaphragm force-generating capacity.

In the present study, we assessed the time course of the effects of 3 days of CMV (passive ventilation) on the diaphragm force-generating capacity in sedated rabbits. We hypothesized that 1) CMV decreases the force-generating capacity of the diaphragm in a time-dependent manner and 2) diaphragm inactivity with CMV produces structural changes contributing to the decrease in diaphragm muscle force.

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METHODS

Animal Preparation and Surgical Procedures

The Research and Development subcommittee on animal studies of the Veterans Affairs Long Beach Health Care System approved the study. We studied 30 adult male New Zealand White rabbits. The animals were grouped randomly and in equal numbers (n = 6) as follows: 1) CMV for 3 days (3d-CMV), 2) CMV for 1 day (1d-CMV), 3) 0 cmH2O continuous positive airway pressure (CPAP) for 3 days (3d-CPAP), 4) 0 cmH2O CPAP for 1 day (1d-CPAP), and 5) surgical control (C).

CMV groups. Time-triggered, pressure-limited ventilation (model 7200ae or 840 Nellcor-Puritan Bennett, Mallinckrodt, Carlsbad, CA) was applied using neonatal circuits. The inspired air was humidified. The inspiratory pressure was set to deliver tidal volume (VT) of 6–8 ml/kg with the inspiratory time equal to that of spontaneous breathing. The ventilator rate was set sufficiently high to suppress the animal’s diaphragm electrical activity (see below) within a range of 40–50 breaths/min. The positive end-expiratory airway pressure was set at 0 cmH2O, and the inspired O2 fraction was set to maintain arterial Po2 at >60 Torr. The animals were euthanized after 3 days or 1 day of CMV.

CPAP groups. To control for the duration of anesthesia and sedation, 0 cmH2O CPAP was applied. As with CMV, the inspired air was humidified. The trigger sensitivity was set at 1 l/min with a base flow of 5 and 2 l/min in the 7200ae and 840 ventilators, respectively. The animals were euthanized after 3 days and 1 day of breathing spontaneously on CPAP.

C group. The animals were euthanized on the same day after surgical procedures as the CMV groups. Before euthanasia, in vivo diaphragm contractile properties were measured (see below).

The surgery was performed using aseptic techniques and consisted of two stages. The first stage was a surgical surgery, in which the animals underwent general anesthesia with an initial dose of ketamine hydrochloride (35 mg/kg) and xylazine (5 mg/kg) administered intramuscularly. A maintenance dose of ketamine hydrochloride (2 mg/kg)-xylazine (0.2 mg/kg) was administered intramuscularly every 20 min as needed. The depth of anesthesia was monitored by the absence of jaw tone. A laparotomy incision was performed for implantation of a pair of wire electrodes (40 gauge; Medwire, Mount Vernon, NY) in the ventral region of the diaphragm adjacent to the central tendon bilaterally. The wires were then tunneled through the skin for later recording of diaphragm electromyogram (EMGdia). The EMGdia signals were amplified and band-pass filtered between 10 and 1,000 Hz (model BMA-830, CWE, Ardmore, PA). A complete suppression of EMGdia during CMV ensured diaphragm inactivity or passive ventilation. The laparotomy incision was closed, and the animals were allowed to recover for ≥7 days (8–28 days) before the second stage of surgery was performed. One-half of the animals in each group underwent the first stage of surgery.

In the second stage of surgery, under general anesthesia, the trachea was cannulated with a tracheostomy tube (4 mm ID, 6 cm long) through a tracheotomy incision. The external jugular vein was cannulated for continuous intravenous fluid infusion (lactated Ringer-dextrose, 100 ml·kg⁻¹·day⁻¹) and intravenous access of medications. The common carotid artery was cannulated for blood pressure (model P23ID, Gould) and heart rate monitoring and arterial blood sampling (0.1 ml) for arterial blood-gas analysis (model iSTAT PCA, i-STAT, East Windsor, NJ) every 12 h and as needed. Arterial blood (2 ml) was also withdrawn for electrolyte measurements once daily. A feeding tube was inserted into the stomach via a small incision in the esophagus. After the surgical procedures, the skin was closed in layers, except for the C group.

Animal Monitoring and Care During CMV and CPAP

During CMV and CPAP, continuous intravenous sedation was maintained with diazepam with a loading dose of 4 mg/kg im. This was followed by a continuous intravenous infusion of 2–5 mg/h titrated to limb movement. The dose of diazepam was inadequate to suppress the respiratory center. In the CMV groups, suppression of diaphragm activities was maintained by adjusting the ventilator settings and administering additional doses of the maintenance ketamine-xylazine mixture. In addition, the following medications were given: buprenorphine (0.05 mg/kg sc) every 12 h for analgesia, atropine sulfate (0.02 mg/kg sc) every 12 h to reduce bronchial secretions, and penicillin G procaine (300,000 U im) every 12 h to prevent infection. To prevent metabolic acidosis, sodium bicarbonate was administered (1–3 meq/h iv), titrated according to arterial pH (31). Potassium chloride and magnesium sulfate were administered through the feeding tube, titrated by the serum electrolytes. Liquid enteral nutrition (F3978SP, BioServ, Frenchtown, NJ) was delivered through the feeding tube at 100 cal·kg⁻¹·day⁻¹ in equal divided doses. Blood pressure, heart rate, and EMGdia were continuously monitored. The analog signals were displayed in real time on a computer monitor of a data acquisition system and recorded every 2 h (WinDag/Pro, Datas Instruments, Akron, OH). Rectal temperature was continuously monitored and maintained at 36–38°C with a heating blanket. Airway pressure, VT, and flow signals were monitored from the ventilator display screen. In the animals for which EMGdia was not available, complete suppression of diaphragm muscle activity during CMV was monitored from the flow and airway pressure signals for inspiratory efforts. To prevent atelectasis, the lungs were inflated with a set inspiratory pressure to produce VT of 15 ml/kg for five consecutive breaths every 15 min in all animals, including the C group. Passive stretches have not been shown to be the major determinant of morphological adaptation to diaphragm inactivity (40). The animals were positioned in various postures (dorsal, ventral, and left and right lateral decubitus) every 2 h. Suctioning of the trachea was performed every 2 h and as needed. The bladder was expressed every 12 h. A physician or an experienced research assistant provided around-the-clock coverage for the duration of the study. All experimental procedures were in strict accordance with the Animal Welfare Act.

In Vivo Measurement of Diaphragm Contractile Properties

After receiving 3 days or 1 day of CMV or CPAP and after surgical procedures in the C group, with the animals under general anesthesia (n = 4 in each group), both phrenic nerves in the neck were isolated for stimulation using bipolar stainless steel wire electrodes (38 gauge; Cooner Wire, Chatsworth, CA). A 16-gauge liquid-filled catheter with multiple holes at the sides was inserted percutaneously into the pleural space for measurements of pleural pressure (Ppl; model DP15-32, Validyne, Northridge, CA). Aspirating the air from the pleural space and simultaneously applying positive pressure at the airway opening evacuated the resulting pneumothorax. A laparotomy incision was made, and the abdominal contents were exposed; hence, Pdi = atmospheric pressure – Ppl. During the surgery, the animals in the C group contin-

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used to breathe spontaneously, and those in the CMV and CPAP groups continued to receive the respective mode of ventilation. A pneumotachograph (no. 000, Fleisch, Lausanne, Switzerland) was attached to the inspiratory line of a miniature two-way nonrebreathing valve (model 2384, Hans Rudolph, Kansas City, MO) connected to the tracheostomy tube. This allowed Pdi measurement at end-expiratory lung volume, with the inspiratory line occluded during supramaximal bilateral phrenic nerve stimulation (0.15-ms pulses in 500-ms train). Current intensity was adjusted until maximum tetanic force (Po, 500-ma train and 75 Hz) responses were obtained. Thereafter, the stimulus intensity was set at 125% of this value. The frequency of stimulation was determined using stimulation frequencies at 5–100 Hz with 1 min between stimuli. Pdi was expressed as a percentage of the average maximum Pdi (at 100 Hz of stimulation) of the C group.

In Vitro Measurement of Diaphragm Contractile Properties

After Pdi measurements, the animals were euthanized with an overdose of pentobarbital sodium (100 mg/kg iv). The diaphragm muscle was rapidly excised from the midcostal region, with the insertion of fibers at the ribs and central tendon intact. The techniques utilized in determining the in vitro isometric contractile and fatigue properties of the diaphragm have been previously reported (33). A diaphragm muscle strip (~5 mm wide) was mounted vertically between two platinum plate electrodes that cover the entire length of the muscle strip in a 26°C bath containing Rees-Simpson solution (pH 7.40) with the following composition (in mM): 135 Na⁺, 5 K⁺, 2 Ca²⁺, 1 Mg²⁺, 120 Cl⁻, 25 HCO₃⁻, and 0.012 d-tubocurarine. The solution was continuously aerated with 95% O₂-5% CO₂. The central tendon of the muscle was glued to a nylon mesh that was then attached to a calibrated force transducer (Grass FT10, Astro-Med, West Warwick, RI), and the rib end of the muscle was clamped to a micromanipulator to allow adjustments of muscle length. The muscle was stimulated using 1.5-ms-duration monophasic rectangular pulses delivered via a current amplifier (Mayo Section of Engineering) that was controlled by a Grass S88 stimulator. Current intensity was adjusted until Po (500-ma train and 50 Hz) responses were obtained. Thereafter, the stimulus intensity was set at 125% of this value. The length at which the muscle produced maximal isometric tension (L₀) was determined. Subsequently, L₀ was measured using digital calipers. At L₀, peak twitch force (P₀), contraction time (CT, i.e., the time to reach peak twitch force), and the time for P₀ to relax to one-half P₀ (RT₁/₂) were determined from a series of contractions induced by single-pulse stimuli. As with Pdi, the dependence of diaphragm force generation on the frequency of stimulation was determined using 1-s-duration trains of stimuli at 5–100 Hz. The interval between each train of stimuli was 1 min. P₀ was determined. All force responses were displayed on a storage oscilloscope and acquired using a data acquisition computer system (WinDaq, Dataq Instruments) at a sampling frequency of 1,000 Hz. Forces were normalized for muscle cross-sectional area (CSA), which was estimated using the following formula:

\[ \text{Muscle mass (g)} / [L₀ \times \text{muscle density (g/cm}^3)] \]

where 1.056 g/cm³ was used for muscle density. Diaphragm muscle fatigability was evaluated with a test described by Burke et al. (4), which employed repetitive stimulation at 40 Hz in trains of 330-ms duration, with one train repeated each second. A fatigue index (FI) was calculated as the ratio of force generated after 2 min of stimulation to the initial force.

Immunohistochemistry for Fiber Classification According to Myosin Heavy Chain Expressions

Two additional segments of diaphragm muscle strip were prepared (~4 × 10 mm) and stretched to 1.5 times the resting excised muscle length (approximate optimal length for muscle force generation) (28). One muscle segment was fixed for electron microscopy (EM) staining (see below). The other muscle segment was rapidly frozen in isopentane cooled to its melting point by liquid nitrogen. Four serial muscle sections were cut at 10 µm by using a cryostat kept at ~20°C (model 2800E Frigocut, Reichert-Jung) and reacted with mouse primary antibodies against different myosin heavy chain (MHC) isoforms. Pairs of mouse IgG or IgM primary antibodies were used, i.e., anti-MHC2A (Novoceastra [IgG]), anti-MHC2B (Blau A4.74 [IgG]) or Blau N1.551 [IgM]), and anti-MHC2X [Schiaffino BFF3 (IgM)], and only a single antibody was used for identification of MHC2X, i.e., anti-MHC2Alx [Schiaffino BF-35]. Primary antibodies were diluted in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (5 mg/ml) and applied to the muscle section for ~2 h at room temperature. Slides were then washed in PBS and reacted with Cy3- or Cy5-conjugated secondary antibodies (goat anti-mouse IgG or goat anti-mouse IgM) for 45 min at room temperature. The use of antibody pairs allowed for double labeling of MHC isoform expression in the same section with minimal cross-reactivity. This was confirmed by adding the opposite secondary antibody to a section incubated with only IgG or IgM primary antibody. Sections incubated with only the secondary antibodies served as controls for nonspecific reactivity of all primary antibodies. The slides were then washed in PBS and imaged with a Bio-Rad (MRC500/600) confocal system mounted on an Olympus (BH2) microscope and equipped with an Ar-Kr laser (41). This allows determination of fiber CSA, proportions, and relative contributions to total diaphragm CSA. The latter was calculated on the basis of the proportions and mean CSA of fibers expressing specific MHC.

Fiber Succinic Dehydrogenase Activity

The quantitative histochemical procedure for measuring fiber succinic dehydrogenase (SDH) activity has been previously described (2, 32). Briefly, the reduction of nitro blue tetrazolium (NBT) to its diformazan (NBT-dfz) was used as the reaction indicator. Four serial 6-μm-thick muscle sections were cut: two sections were reacted with succinate added to the incubation medium and two sections without succinate to control for nonspecific reduction of NBT. The concentration of NBT-dfz ([NBT-dfz]) deposited within a fiber during the SDH reaction was determined from optical density (OD) measurements using the Beer-Lambert equation: [NBT-dfz] = OD/kl, where fiber OD was measured at 570 nm (the peak absorbance wavelength for NBT-dfz), k was the molar extinction coefficient for NBT-dfz (26,478 mol⁻¹·cm⁻¹), and l was the path length for light absorbance (6 μm). From these measurements, the maximum velocity of the SDH reaction was determined, and the mean SDH activity of each fiber was expressed as millimoles fumarate per liter of tissue per minute. The SDH activities of ~125 fibers were analyzed in each muscle sample.

Determination of MHC Isoform Composition

Myosin was extracted from the muscle segments by scissor mincing in a high-salt solution (in mM: 300 NaCl, 100 NaH₂PO₄, 50 Na₂HPO₄, 1 Na₃PO₄, and 10 EDTA, pH 6.5) at 4°C for 4 min (5). Extracts were centrifuged, and the super-
natants were recovered. Ten microliters of supernatant were diluted (1:10) in a low-salt buffer consisting of 1 mM EDTA and 0.1% 2-mercaptoethanol (vol/vol) and stored overnight at 4°C to allow precipitation of myosin filaments. The filament solution was subsequently centrifuged to form a pellet, which was then dissolved in myosin sample buffer (0.5 M CaCl2 and 10 mM NaH2PO4) and then diluted (1:200) in SDS sample buffer [62.5 mM Tris-HCl, 2% (wt/vol) SDS, 10% glycerol, 5% (vol/vol) 2-mercaptoethanol, and 0.001% (wt/vol) bromophenol blue, pH 6.8]. The samples were boiled for 2 min and stored at -80°C. Different MHC isoforms were separated by SDS-PAGE. Gel preparation was based on modification of the procedure by Sugiuira and Murakami (34). A 3.5% acrylamide concentration (pH 6.8) was used in the stacking gel, and the separating gel (8 × 10 cm, 0.75 mm thick, Hoefer SE250) consisted of a gradient of 5–8% acrylamide (pH 8.8) with 25% (vol/vol) glycerol. All samples were run at a constant current of 20 mA/gel until the tracking dye reached the bottom of the gel. After completion of the gel run, the gels were removed from the plates and silver stained according to the procedure of Oakley et al. (27).

The relative expression of different MHC isoforms was then quantified by densitometry.

**Determination of Myofibril Injury**

EM was performed on the stretched diaphragm muscle segment in five animals from each group and on the soleus muscle in two animals from the 3d-CMV, 3d-CPAP, and C groups. The muscle was fixed as described by Mathieu-Costello et al. (23). The muscle was immersed for 1 h in 6.5% glutaraldehyde in a 0.1 M sodium cacodylate buffer (total osmolarity of the fixative = 1,100 mosM, pH 7.4). The muscle was then cut into 2-mm pieces and reimmersed in the same solution for an additional 1 h. After fixation, the tissue was washed three times for 10 min each in 0.1 M sodium cacodylate buffer (pH 7.4). The tissues were postfixed in 1.0% OsO4 and 1.0% potassium ferrocyanate in the same buffer for 4 h. The tissues were then washed in three changes of distilled water for 10 min each. Dehydration followed through a graded series of ethanol; then the tissue was infiltrated with propylene oxide twice for 10 min each, 50% Araldite-50% propylene oxide for 2 h, and 100% Araldite for 24 h and polymerized at 65°C for 2 days. Sections (1 μm thick) were cut on an LKB Ultratome III using a diamond knife and stained with 0.25% toluidine blue solution. After muscle fibril orientation was determined with light microscopy, blocks were reoriented, and ultrathin sections (50–70 nm) were cut transversely to the muscle fiber axis. These sections were contrasted with uranyl acetate and bismuth subnitrate for transmission EM.

The magnitude of myofibril injury was estimated by determining the volume density (Vv) of normal or abnormal myofibrils and mitochondria per volume of muscle fiber from the EM transverse section samples at a final magnification of ×24,000 (23, 37). The pathologist working on the EM images was uninformed about the nature of the study. Ten direct positive micrographs were obtained by systematic sampling in one ultrathin section from each block. Two randomly chosen blocks per sample (i.e., a total of 20 micrographs) were analyzed. The micrographs were scanned using a film scanner (Polaroid Sprint Scan 45i, Meyer Instruments, Houston, TX), and the image was subsequently projected on a computer screen. A 140-point square grid was superimposed on the image using image analysis software (Image Pro Plus version 4.0, Media Cybernetics, Silver Spring, MD). Points that fall into the image were assigned as follows: 1) normal myofibrils, 2) abnormal disrupted myofibrils, 3) normal myo-

**Lung Infection Surveillance**

Because sepsis has been shown to cause respiratory muscle dysfunction (19), in the first two animals in the 3d-CMV and 3d-CPAP groups, after euthanasia, a randomly sampled piece of tissue was obtained from the right lower lobe of the lung for culture and histological examination. Because there was no bacterial growth or inflammatory cell infiltration under light microscopy, these measures were not performed in subsequent experiments. In general, the development of pneumonia was associated with hypoxemia, wide pulse pressure, hypertension, and sudden death before the completion of the experiments. Only animals completing the experiments were included in the data analysis.

**Statistics and Data Analysis**

Values are means ± SE unless specifically indicated. A three-way ANOVA (SigmaStat, version 2.03, SPSS Science, Chicago, IL) was used to compare Pdi and diaphragm force among groups using the grouping variables of frequency of stimulation, days on mechanical ventilation (0, 1, and 3 days), and mode of ventilation (CPAP and CMV). Where appropriate, the latter two grouping variables were also used in a two-way ANOVA for comparison of other variables among groups. When the F value was significant, post hoc analysis was performed using Tukey's test for pairwise multiple comparisons. A linear regression was used to determine the contribution of diaphragm muscle injury to diaphragm muscle force-generating capacity. Group differences and linear regression were considered significant when P ≤ 0.05.

**RESULTS**

The animals' average body weight was comparable among all experimental groups: 3.8 ± 0.5, 3.8 ± 0.3, 3.9 ± 0.6, 3.9 ± 0.6, and 3.8 ± 0.3 (SD) kg for 3d-CMV, 3d-CPAP, 1d-CMV, 1d-CPAP, and C, respectively. None of the animals lost weight (data not shown). For the duration of the study, the total amount of anesthetics (mg/kg body wt) and diazepam (mg/h) administered was not significantly different among groups (Table 1). The 3d-CMV group tended to receive a larger total dose of anesthetics and diazepam than the 3d-CPAP group. This was expected to ensure a complete suppression of diaphragm muscle activity. An average of three to four brief episodes of breakthrough diaphragm activity per day occurred during CMV. The amount of sodium bicarbonate (meq/h) the animals received to prevent metabolic acidosis was also similar among groups (Table 1).

Figure 1 shows the trend of arterial pH, arterial PCO2 (Paco2), and bicarbonate. At a given time, arterial pH, Paco2, and bicarbonate were similar among or between groups, except at 12 h, when Paco2 and bicarbonate were higher in the 1d-CPAP group than in the 1d-CMV group.
Dependency of $P_{di}$ on Frequency of Stimulation

At all stimulation frequencies, $P_{di}$ was significantly reduced in the 3d-CMV group compared with the 3d-CPAP group ($P < 0.01$; Fig. 2). At a stimulation frequency of 100 Hz, $P_{di}$ decreased to 63% in the 1d-CMV group ($P < 0.05$) and decreased further to 49% in the 3d-CMV group ($P < 0.01$) compared with the C group [76.9 ± 19.3 (SE) cmH$_2$O].

Diaphragm Isometric Contractile and Fatigue Properties

At a similar $L_0$, peak twitch and tetanic tensions were significantly reduced in the 3d-CMV group compared with the 3d-CPAP, 1d-CMV, and C groups (Table 2). Compared with the C group, diaphragm force-generating capacity did not decline significantly in the 1d-CMV group. Twitch CT and RT$_{1/2}$ tended to increase in the 3d-CMV group, whereas FI was not significantly different among the groups (Table 2). Figure 3 demonstrates the force-frequency relationship of all groups. At all stimulation frequencies, force in the 3d-CMV group was significantly reduced compared with the 3d-CPAP, 1d-CMV, and C groups. At a stimulation frequency of 100 Hz, the diaphragm force declined to 86% in the 1d-CMV group (not significant) and to 44% in the 3d-CMV group ($P < 0.01$).

Fiber CSA, SDH Activity, and MHC Isoform Expression

CSA of fibers expressing the various MHC isoforms did not change significantly across experimental groups. In the 3d-CMV group, we did not detect fibers expressing MHC$_{2X}$ in three of the five animals studied. In the remaining two animals in which fibers expressing MHC$_{2X}$ were identified, there was no evidence of atrophy (Table 3). Proportions of fibers expressing MHC$_{slow}$, MHC$_{2A}$, and MHC$_{2X}$, its relative contribution to total CSA of the diaphragm, and SDH enzyme activity were similar among all groups. In the 3d-CMV group, although FI tended to increase (Table 2), this

![Fig. 1. Trend of arterial pH, arterial $P_{CO_2}$ ($P_{CO_2}$), and bicarbonate ($HCO_3^-$) in control animals and animals subjected to 0 cmH$_2$O continuous positive airway pressure (CPAP) for 1 day (1d-CPAP) and 3 days (3d-CPAP) and controlled mechanical ventilation (CMV) for 1 day (1d-CMV) and 3 days (3d-CMV). Values are means ± SE; $n = 6$ animals. *$P < 0.01$; †$P < 0.05$: 1d-CPAP vs. 1d-CMV.]

![Fig. 2. Transdiaphragmatic pressure ($P_{di}$) at various stimulation frequencies in control, 1d-CPAP, 3d-CPAP, 1d-CMV, and 3d-CMV groups. $P_{di}$ was expressed as percentage of average maximum $P_{di}$ ($P_{dimax}$) of the control group. Values are means ± SE; $n = 4$ animals. *$P < 0.01$, 3d-CMV vs. 3d-CPAP (at all stimulation frequencies) and 1d-CMV vs. control (100 Hz); †$P < 0.05$, 3d-CMV vs. 1d-CMV (100 Hz).]

Table 1. Doses of anesthetic, sedative drugs, and sodium bicarbonate

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>1d-CPAP</th>
<th>1d-CMV</th>
<th>3d-CPAP</th>
<th>3d-CMV</th>
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<tr>
<td>Ketamine, mg/kg body wt</td>
<td>12.7 ± 0.6</td>
<td>16.2 ± 3.2</td>
<td>17.7 ± 4.0</td>
<td>21.2 ± 5.3</td>
<td>36.2 ± 14.1</td>
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<tr>
<td>Xylazine, mg/kg body wt</td>
<td>1.2 ± 0.2</td>
<td>1.3 ± 0.3</td>
<td>2.0 ± 0.4</td>
<td>2.2 ± 0.6</td>
<td>3.5 ± 1.5</td>
</tr>
<tr>
<td>Diazepam, mg/h</td>
<td>3.2 ± 0.3</td>
<td>3.2 ± 0.3</td>
<td>3.4 ± 0.4</td>
<td>3.9 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Sodium bicarbonate, meq/h</td>
<td>0.5 ± 0.3</td>
<td>1.7 ± 0.5</td>
<td>1.7 ± 0.4</td>
<td>1.6 ± 0.4</td>
<td>1.0 ± 0.2</td>
</tr>
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</table>

Values are mean ± SE; $n = 6$ animals in each group. Values for ketamine, xylazine, and diazepam reflect total maintenance dose and do not include initial or loading dose. 1d-CPAP and 3d-CPAP, 1 day and 3 days of 0 cmH$_2$O continuous positive airway pressure of respectively; 1d-CMV and 3d-CMV, 1 day and 3 days of controlled mechanical ventilation, respectively.
increase was associated with the opposite trend in SDH activity (Table 3). Fatigue resistance would predictably be coupled with elevated oxidative enzyme capacity. This discordance is unclear, because the animal in which SDH activity cannot be measured because of freezing artifact had a low FI of 31%. The injury explained 66% of the variance in the reduction of diaphragm force-generating capacity due to diaphragm inactivity is likely multifactorial. These factors include problems in excitation-contraction coupling [a decrease in sarcoplasmic reticulum (SR) Ca$^{2+}$ release capacity] (18, 42), myofibrillar atrophy, decreased myofibrillar protein concentration (13), and myofibrillar injury.

Our study was not designed to assess the effect of inactivity on diaphragm muscle sarcolemma resting membrane action potential or on SR Ca$^{2+}$ release capacity. Resting action membrane potential has been reported to decrease (24) or remain unchanged (10) with skeletal muscle inactivity. However, the effect of CMV on diaphragm resting membrane action potential is unknown. Diaphragm inactivity in hamsters induced by phrenic denervation or tetrodotoxin blockade was associated with a decrease in SR Ca$^{2+}$ release (18) with prolongation in CT (42). In the present study, although CT in the 3d-CMV group was not significantly different from that in the 3d-CPAP group, it tended to increase (Table 2), suggesting that CMV might alter SR Ca$^{2+}$ release. The tendency of RT/$V_o$ to increase also suggests that SR Ca$^{2+}$ uptake capacity might have been impaired.

DISCUSSION

The present study is the first to demonstrate that 1) the reduction in diaphragm force-generating capacity with CMV is time dependent in the intact animal and in vitro preparation, 2) diaphragm muscle inactivity was associated with diaphragm muscle injury, and 3) the injury accounts for the reduction in diaphragm force.

The present study differs from previous studies (1, 21), in that the control group consisted of spontaneously breathing animals (1d-CPAP and 3d-CPAP groups) receiving similar total amounts of anesthesia and sedative drugs for the same duration as the experimental group; therefore, the reduced diaphragm force was primarily related to the effect of diaphragm inactivity. Furthermore, the animals did not receive continuous administration of anesthesia (thiopental sodium), as in the study of Le Bourdelles et al. (21), or paralyzing agent (pancuronium bromide), as in the study of Anzueto et al. (1). Barbiturates (36) and pancuronium bromide (14) have direct adverse effects on skeletal muscle function.

**Ultrastructure of the Diaphragm and Soleus**

In contrast to the C and 3d-CPAP groups, myofibrillar damage was evident in the 3d-CMV group (Fig. 4). The $V_o$ of abnormal myofibrils of the diaphragm was significantly higher in the 3d-CMV group than in the 1d-CPAP group (P < 0.01; Table 3). In addition, ultrastructural changes observed in the diaphragm were not seen in the soleus muscle of the same animals (n = 2; Fig. 5). When $P_o$ was correlated with the $V_o$ of abnormal myofibrils, diaphragm muscle injury explained 66% of the variance in the reduction of tetanic force ($r = 0.82$, $P < 0.01$; Fig. 6).

**DISCUSSION**

The present study is the first to demonstrate that 1) the reduction in diaphragm force-generating capacity with CMV is time dependent in the intact animal and in vitro preparation, 2) diaphragm muscle inactivity was associated with diaphragm muscle injury, and 3) the injury accounts for the reduction in diaphragm force.

The present study differs from previous studies (1, 21), in that the control group consisted of spontaneously breathing animals (1d-CPAP and 3d-CPAP groups) receiving similar total amounts of anesthesia and sedative drugs for the same duration as the experimental group; therefore, the reduced diaphragm force was primarily related to the effect of diaphragm inactivity. Furthermore, the animals did not receive continuous administration of anesthesia (thiopental sodium), as in the study of Le Bourdelles et al. (21), or paralyzing agent (pancuronium bromide), as in the study of Anzueto et al. (1). Barbiturates (36) and pancuronium bromide (14) have direct adverse effects on skeletal muscle function.

**Influence of CMV on Diaphragm Contractile Properties**

The mechanism for the reduced diaphragm force-generating capacity due to diaphragm inactivity is likely multifactorial. These factors include problems in excitation-contraction coupling [a decrease in sarcoplasmic reticulum (SR) Ca$^{2+}$ release capacity] (18, 42), myofibrillar atrophy, decreased myofibrillar protein concentration (13), and myofibrillar injury.

Our study was not designed to assess the effect of inactivity on diaphragm muscle sarcolemma resting membrane action potential or on SR Ca$^{2+}$ release capacity. Resting action membrane potential has been reported to decrease (24) or remain unchanged (10) with skeletal muscle inactivity. However, the effect of CMV on diaphragm resting membrane action potential is unknown. Diaphragm inactivity in hamsters induced by phrenic denervation or tetrodotoxin blockade was associated with a decrease in SR Ca$^{2+}$ release (18) with prolongation in CT (42). In the present study, although CT in the 3d-CMV group was not significantly different from that in the 3d-CPAP group, it tended to increase (Table 2), suggesting that CMV might alter SR Ca$^{2+}$ release. The tendency of RT/$V_o$ to increase also suggests that SR Ca$^{2+}$ uptake capacity might have been impaired.

**Table 2. In vitro diaphragm contractile properties**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>1d-CPAP</th>
<th>1d-CMV</th>
<th>3d-CPAP</th>
<th>3d-CMV</th>
</tr>
</thead>
<tbody>
<tr>
<td>$L_{o, cm}$</td>
<td>3.0 ± 0.2</td>
<td>2.8 ± 0.2</td>
<td>2.7 ± 0.1</td>
<td>2.7 ± 0.2</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>CT, ms</td>
<td>70.8 ± 8.5</td>
<td>65.0 ± 5.5</td>
<td>72.5 ± 6.8</td>
<td>69.2 ± 6.2</td>
<td>87.5 ± 14.8</td>
</tr>
<tr>
<td>RT/$V_o$, ms</td>
<td>92.5 ± 18.7</td>
<td>85.8 ± 14.4</td>
<td>95.0 ± 15.1</td>
<td>95.0 ± 9.9</td>
<td>126.7 ± 25.4</td>
</tr>
<tr>
<td>$P_o$/CSA, N/cm²</td>
<td>7.5 ± 1.1</td>
<td>7.7 ± 0.6</td>
<td>6.5 ± 0.7</td>
<td>7.6 ± 1.1</td>
<td>2.8 ± 0.6*</td>
</tr>
<tr>
<td>$P_o$/CSA, N/cm²</td>
<td>18.8 ± 1.6</td>
<td>17.5 ± 1.2</td>
<td>16.7 ± 0.6</td>
<td>18.9 ± 1.2</td>
<td>8.5 ± 1.2*</td>
</tr>
<tr>
<td>FI, %</td>
<td>38.2 ± 4.9</td>
<td>40.1 ± 2.9</td>
<td>38.9 ± 2.4</td>
<td>49.8 ± 3.7</td>
<td>51.7 ± 6.9</td>
</tr>
</tbody>
</table>

Values are mean ± SE; n = 6 animals in each group. $L_{o, cm}$ length at which diaphragm muscle strip produced maximal isometric tension; CT, contraction time from onset of muscle contraction to peak twitch force ($P_t$); RT/$V_o$ time for $P_o$ to relax to one-half of $P_o$; $P_o$, maximum tetanic force; CSA, cross-sectional area (see METHODS for calculation); FI, fatigue index. *$P < 0.01$, 3d-CMV vs. 3d-CPAP, 1-d CMV, and control.

**Fig. 3. Diaphragm muscle force-generating capacity at various stimulation frequencies in control, 1d-CPAP, 3d-CPAP, 1d-CMV, and 3d-CMV groups. Values are means ± SE; n = 6 animals.** *$P < 0.01$, 3d-CMV vs. 3d-CPAP, 1d-CMV, and control. CSA, cross-sectional area.
The decrease in diaphragm muscle force in the 3d-CMV group was not related to myofibril atrophy, since CSA was unchanged for all fibers expressing the various MHC isoforms among groups (Table 3). For the same duration of diaphragm inactivity, our findings were at variance with those of Gosselin and co-workers (15). After 3 days of phrenic denervation, hypertrophy across all fiber types was observed. The discrepancy between our study and that of Gosselin and co-workers is likely related to differences in the diaphragm inactivity model (Table 4) and prior activation history of the muscle. Indeed, morphological adaptation to dia-

Table 3. Diaphragm muscle fiber CSA, proportion, relative contribution, SDH activity, and MHC isoform expression

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>1d-CPAP</th>
<th>1d-CMV</th>
<th>3d-CPAP</th>
<th>3d-CMV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fiber CSA, μm²</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MHCslow</td>
<td>1,536 ± 186</td>
<td>1,443 ± 79</td>
<td>1,510 ± 84</td>
<td>1,245 ± 73</td>
<td>1,427 ± 117</td>
</tr>
<tr>
<td>MHC2A</td>
<td>2,208 ± 207</td>
<td>1,850 ± 179</td>
<td>2,354 ± 187</td>
<td>1,801 ± 270</td>
<td>2,031 ± 309</td>
</tr>
<tr>
<td>MHC2X</td>
<td>2,446 ± 233</td>
<td>2,583 ± 233</td>
<td>2,930 ± 370</td>
<td>2,359 ± 272</td>
<td>2,406 ± 64†</td>
</tr>
</tbody>
</table>

Fiber proportion, %

|                |         |         |        |         |        |
| MHCslow        | 43.8 ± 2.6 | 48.2 ± 2.0 | 48.9 ± 4.6 | 43.3 ± 3.7 | 48.9 ± 4.3 |
| MHC2A          | 43.5 ± 3.0 | 39.5 ± 1.0 | 43.8 ± 4.3 | 45.8 ± 3.3 | 45.0 ± 1.9 |
| MHC2X          | 12.7 ± 2.6 | 12.2 ± 1.2 | 7.3 ± 1.2 | 10.9 ± 2.5 | 4.2 ± 2.6† |

Relative contribution, %

|                |         |         |        |         |        |
| MHCslow        | 7.0 ± 0.6 | 6.0 ± 0.7 | 6.0 ± 0.6 | 6.4 ± 0.3 | 5.3 ± 0.6 |
| MHC2A          | 3.4 ± 0.5 | 3.5 ± 0.8 | 3.0 ± 0.4 | 3.8 ± 0.6 | 2.8 ± 0.6 |
| MHC2X          | 1.9 ± 0.2 | 1.6 ± 0.3 | 1.9 ± 0.3 | 1.6 ± 0.4 | 0.4 ± 0.2† |

SDH activity, mmol fumarate·l tissue⁻¹·min⁻¹

|                |         |         |        |         |        |
| MHCslow        | 26.4 ± 1.7 | 27.6 ± 0.5 | 32.0 ± 0.7 | 28.4 ± 1.0 | 29.6 ± 2.2 |
| MHC2A          | 62.2 ± 1.1 | 59.8 ± 1.2 | 57.2 ± 0.9 | 61.0 ± 1.9 | 66.4 ± 1.9* |
| MHC2X          | 11.4 ± 0.9 | 12.4 ± 1.0 | 10.8 ± 0.4 | 10.6 ± 1.3 | 4.0 ± 2.5† |

MHC isoform expressions, %

|                |         |         |        |         |        |
| MHCslow        | 26.4 ± 1.7 | 27.6 ± 0.5 | 32.0 ± 0.7 | 28.4 ± 1.0 | 29.6 ± 2.2 |
| MHC2A          | 62.2 ± 1.1 | 59.8 ± 1.2 | 57.2 ± 0.9 | 61.0 ± 1.9 | 66.4 ± 1.9* |
| MHC2X          | 11.4 ± 0.9 | 12.4 ± 1.0 | 10.8 ± 0.4 | 10.6 ± 1.3 | 4.0 ± 2.5† |

Values are means ± SE; n = 5 animals. SDH, succinic dehydrogenase; MHC, myosin heavy chain. *P < 0.01 vs. 1d-CMV. † Fibers expressing MHC2X were detected in only 2 of the 5 animals.

Morphological and Metabolic Properties and MHC Isoform Expressions

The decrease in diaphragm muscle force in the 3d-CMV group was not related to myofibril atrophy, since CSA was unchanged for all fibers expressing the various MHC isoforms among groups (Table 3). For the same duration of diaphragm inactivity, our findings were at variance with those of Gosselin and co-workers (15). After 3 days of phrenic denervation, hypertrophy across all fiber types was observed. The discrepancy between our study and that of Gosselin and co-workers is likely related to differences in the diaphragm inactivity model (Table 4) and prior activation history of the muscle. Indeed, morphological adaptation to dia-

Fig. 4. Electron-microscopic cross sections of diaphragm myofibrils of a control (A), a 3d-CPAP (B), and a 3d-CMV (C) animal. Myofibril damage was evident in 3d-CMV group.
Diaphragm inactivity depends on intact respiratory center-phrenic motoneuron transmission and neurotrophic influence, with the predominant effect being neurotrophic influence (25). Morphological adaptation was attenuated in diaphragm inactivity due to C2 spinal cord hemisection compared with denervation or tetrodotoxin blockade (25, 41). In all three models of diaphragm inactivity, hypertrophy of type I fibers was observed; however, in C2 hemisection, the hypertrophy was significantly less than with denervation or tetrodotoxin blockade, whereas the CSA of type II fibers was unchanged. In diaphragm inactivity due to CMV, respiratory center-phrenic motoneuron transmission and neurotrophic influence were intact, and the CSA of fibers expressing MHCslow, MHC2A, and MHC2X was unchanged compared with the control group. Similarly, 3 days of CMV did not affect the fiber proportions or their relative contribution to the total CSA. Morphological adaptation of skeletal muscle also depends on prior activation history of the muscle (41). In the present study, we used sedated rabbits with a respiratory frequency of 50 breaths/min. This contrasts with rats used in the study of Gosselin and co-workers, in which the respiratory frequency under general anesthesia was 80 breaths/min (21) and would have been greater while the animals were awake.

As with morphological properties, in the 3d-CMV group, the reduction in diaphragm force cannot be explained by changes in MHC isoform expressions. In the 3d-CMV group, MHC2X isoforms were undetected in three of the five animals (Table 3). The reason for this observation was unclear, but it is possible that if the duration of CMV was prolonged beyond 3 days, pronounced morphological, metabolic, and MHC adaptations might have been observed, with a shift toward fibers with a higher oxidative capacity and fatigue resistance.

A decrease in myofibril protein concentration may contribute to the decrease in diaphragm force after 3 days of CMV. In the denervated diaphragm muscle, Geiger et al. (13) demonstrated the decrease in MHC content of fast-twitch fibers. The decrease in MHC protein contributed to the decrease in specific force. Similarly, after 3 days of hindlimb immobilization in rats, the rate of myofibril protein synthesis decreased and the rate of protein degradation increased (22). The opposite directions of myofibril protein dynamics may contribute to the reduced diaphragm force we observed in the present study.

Diaphragm Inactivity and Myofibril Injury

The myofibril injury observed after 3 days of CMV was consistent with diaphragm inactivity observed after 3 days of phrenic denervation (15) and in the immobilized hindlimb muscle (20). In the hindlimb muscle, mitochondrial swelling was detected as early as 10 h and myofibril disruption at 36 h of inactivity. Most importantly, in the present study, myofibril injury contributes to the reduced diaphragm force-generating capacity (Fig. 6). The mechanism for the development of myofibril injury with inactivity is unclear. Hypoxemia or ischemia is known to cause mitochondrial and myofibril injuries, and the latter may be mediated via release of oxygen radicals (16). However, oxygenation and blood pressure have been maintained throughout the study. Nonetheless, decreased regional diaphragm perfusion has been demonstrated during CMV. In anesthetized dogs, diaphragm blood flow was significantly reduced by passive mechanical ventilation compared with spontaneous breathing (3). This was more pronounced in the midcostal region, portions of the diaphragm removed for examination of contractile and ultrastructural properties. Although the reduced blood flow with CMV is part of diaphragm blood flow with CMV is part of diaphragm blood flow.
flow autoregulation, cyclic passive shortening was maintained, causing conceivably regional ischemia and myofibril injury. Because of the breakthrough diaphragm activity during CMV, we cannot exclude the possibility of intermittent nonsynchronized contractions, a form of eccentric contraction that may damage myofibril contractile and cytoskeletal components (12).

Despite the reduced diaphragm force, could the animals sustain spontaneous breathing when mechanical ventilation is discontinued? On the basis of our laboratory’s previous study (30), the diaphragm force required to maintain spontaneous breathing was 11% of its maximum. With use of the $P_o$ of the C group, this translated to 2.1 N/cm². Given the FI and $P_o$ of each group, the residual force after a fatiguing maneuver can be calculated as the product of FI and $P_o$ (Fig. 7). As shown in Fig. 7, after 3 days of CMV, notwithstanding the markedly reduced force after fatiguing contractions, the diaphragm muscle force reserve remains adequate to sustain spontaneous breathing.

Critique of Methods

The present study evaluated the effect of CMV, rather than assist-control, a mode more frequently used in clinical practice (8). However, not uncommonly, patients with acute respiratory distress syndrome receive passive ventilation through sedation with or without paralysis during mechanical ventilation (29). Thus we believe that the choice of CMV in this study is in accordance with clinical practice.

During CMV, because EMG$_{dia}$ was monitored in only three of the six animals in each group, passive ventilation might not have been achieved in the remainder of the animals, and it is possible that animal-ventilator asynchrony caused eccentric contractions and diaphragm muscle injury. In the animals without EMG$_{dia}$, we monitored inspiratory efforts from the flow and airway pressure signals. Changes in flow and airway pressure waveforms that suggested inspiratory efforts have been shown to coincide with EMG$_{dia}$ activity (9). The uniform flow and airway pressure waveforms, together with the consistency of respiratory frequency with the set frequency, indicate that passive ventilation had been achieved. However, an unavoidable breakthrough diaphragm activity occasionally occurred and necessitated suppression with an additional dose of anesthesia and sedatives.

In the present study, it was necessary to infuse sodium bicarbonate throughout the experiment to prevent acidemia and profound metabolic acidosis, despite stable hemodynamics. We measured lactic acid levels in four rabbits in each group. Mean lactic acid levels at the end of the experiments were 1.5, 5.4, 6.4, 4.0, and 4.2 mmol/l in C, 1d-CPAP, 1d-CMV, 3d-CPAP, and 3d-CMV groups, respectively ($P < 0.05$ for C vs. other groups). The development of lactic acidosis was not entirely clear. Ketamine administration as an anesthetic agent (35, 38) and/or respiratory alkalosis (Fig. 1) (7) have been implicated in lactic acid production. However, in the C group, lactic acid level was significantly less, despite administration of a similar amount of ketamine (Table 1).

Metabolic acidosis can potentially impair diaphragm muscle contractility (11, 17). However, Coast and co-workers (6) demonstrated that, in a diaphragm muscle strip preparation bathed in lactic acid-buffered medium, diaphragm muscle force did not decrease at pH >6.8. Similarly, Yanos et al. (39) measured the phrenic-stimulated $P_{di}$ in dogs with respiratory or lactic acidosis. $P_{di}$ decreased in the animals with respiratory acidosis but not in those with lactic acidosis. Hence, it is rather unlikely that lactic acidosis contributed to the reduced diaphragm force-generating capacity in the 1d-CMV and 3d-CMV groups. Most importantly, the lactic acid levels in the CPAP groups were similar to those in the CMV groups.

Myofibril injury was scattered, and sampling problems may influence its determination. However, the pathologist performing the cuts and images for EM was not informed about the exact nature of the study; hence, it was unlikely that we underestimated myofibril injury in the other experimental groups.

In summary, CMV had a time-dependent detrimental effect on the diaphragm. The reduced force-generating capacity was partly attributable to myofibril in-

Table 4. Respiratory center-phrenic motoneuron transmission and neurotrophic influence in various models of diaphragm muscle inactivity

<table>
<thead>
<tr>
<th>Respiratory Center-Phrenic Motoneuron Transmission</th>
<th>Neurotrophic Influence</th>
<th>Diaphragm Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phrenic denervation</td>
<td>Disrupted</td>
<td>Inactive</td>
</tr>
<tr>
<td>Tetrodotoxin blockade</td>
<td>Intact</td>
<td>Disrupted</td>
</tr>
<tr>
<td>C2 spinal cord section</td>
<td>Disrupted</td>
<td>Intact</td>
</tr>
<tr>
<td>CMV</td>
<td>Disrupted</td>
<td>Intact</td>
</tr>
</tbody>
</table>
jury. Despite the significantly reduced force, the diaphragm is still capable of sustaining spontaneous breathing. It is unknown whether a mode of mechanical ventilation in which partial diaphragm muscle activity is maintained (e.g., assist-control or pressure support mechanical ventilation) will preserve diaphragm force-generating capacity. Our study was limited to 3 days, and it is unclear whether >3 days of CMV will be associated with persistent injury or recovery.

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