

EMD 57033 partially reverses ventilator-induced diaphragm muscle fibre calcium desensitisation

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Abstract In critically ill patients, ventilator-induced diaphragm muscle fibre dysfunction (VIDD) contributes to weaning problems, increasing hospitalisation time and related costs. VIDD pathophysiology remains partially unknown, especially the characterisation of the contractile dysfunction. In the present study, it was hypothesised that Ca^{2+} activation is affected during VIDD. Ca^{2+} sensitivity of contraction was therefore evaluated at the single skinned diaphragm muscle fibre level in piglets randomised into sham operation or 5-day mechanical ventilation. Ca^{2+} sensitivities of force and stiffness in fibres were significantly impaired in all mechanically ventilated piglets compared with sham-operated controls, suggesting a less efficient Ca^{2+} activation of cells, i.e. a lower relative number of strongly attached cross-bridges for each sub-maximal concentration of Ca^{2+} . In an attempt to test

whether this negative effect of VIDD is reversible, single muscle fibres were exposed to the EMD 57033 Ca^{2+} sensitiser. EMD 57033 (30 μM) improved the Ca^{2+} sensitivity of force and stiffness in fibres from animals that were mechanically ventilated for 5 days as well as in sham-operated piglets. Thus, EMD 57033 partly restored the Ca^{2+} activation of cells, reducing VIDD. This finding offers a strong basis for evaluating the effect of Ca^{2+} sensitizers on diaphragm function in vivo.

Keywords Mechanical ventilation · Diaphragm muscle · Porcine model · Skinned fibre · Ca^{2+} activation · Ca^{2+} sensitizer

Introduction

Approximately 40% of intensive care unit patients are mechanically ventilated for a median duration of 5 to 7 days [4]; 20% to 30% of these patients experience weaning problems [5]. Weaning failure arises from various factors [40], but is commonly associated with ventilator-induced diaphragm muscle fibre dysfunction (VIDD) [11, 39]. VIDD is characterised by severe fibre atrophy and disrupted contractile function caused by a decrease in fibre maximal force production normalised to cross-sectional area (CSA) [16, 29], i.e. specific force. It is unknown whether other important contractile features are also disrupted, making the knowledge of VIDD pathophysiology incomplete.

In the diaphragm, 18 h of mechanical ventilation is sufficient to induce a down-regulation of myofibrillar proteins synthesis rate [33] and up-regulation of myofibrillar protein degradation [31, 34]. The time course of these changes is faster than in limb muscles where 96 h of unloading is

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required to induce similar modifications [28]. The generation of reactive oxygen species during mechanical ventilation has an additional damaging effect on existing proteins, including myosin and thin filament elements [43]. Since specific force as well as Ca^{2+} activation are contractile parameters notably dependent on the proteins content, i.e. myosin [26] and thin filament proteins [23], and on their oxidation status [14, 36], the first aim of the present study was to test the hypothesis that Ca^{2+} sensitivity of contraction is disrupted in VIDD. The skinned fibre preparation has therefore been used to compare contractile function in single diaphragm muscle fibres from sham-operated control piglets and from piglets mechanically ventilated for 5 days. Force and stiffness were measured at varying Ca^{2+} concentrations ($p\text{Cas}$), ranging from 9.0 to 4.5, to study relative force– $p\text{Ca}$ and stiffness– $p\text{Ca}$ relationships and calculate Ca^{2+} sensitivities of force and stiffness. Because of fibre type-specific contractile behaviour, muscle cells were characterised according to their myosin heavy chain (MyHC) isoform expression. Findings of rightward shifts of the relative force– $p\text{Ca}$ and stiffness– $p\text{Ca}$ relationships in fibres expressing the type I and IIa MyHC isoforms confirmed the initial assumption and emphasised the complexity of VIDD pathophysiology.

To date, there is no specific treatment to eradicate or minimise VIDD. Nevertheless, the second objective of the present study was to evaluate the hypothesis that reversing the altered contractile function in VIDD is possible. The use of Ca^{2+} -sensitising agents is a well-recognised effective strategy to restore Ca^{2+} activation in various conditions [27, 37] and may therefore represent one potential pharmacological intervention strategy in VIDD since Ca^{2+} sensitisers such as EMD 57033 bind to myofibrillar proteins, commonly thin filament structures, promoting their Ca^{2+} -induced conformation, subsequent cross-bridge formation and overall contractile activation in cardiac [35, 38] and skeletal muscles [13, 18, 27]. Skinned diaphragm muscle fibres from mechanically ventilated piglets were therefore exposed to 30 μM of EMD 57033 to evaluate the effects on the Ca^{2+} sensitivity of force and stiffness. The leftward shifts of the relative force– $p\text{Ca}$ and stiffness– $p\text{Ca}$ curves particularly in type I fibres corroborated the hypothesis.

Materials and methods

Animals

A detailed description of animals has been given elsewhere [25]. Briefly, eight female domestic piglets (23–30 kg body weight) were included. After initial preparation, animals were allowed to rest for 1 h. Prior to induction of anaesthesia, animals were given an intramuscular (i.m.)

injection of atropine 0.1 mg kg^{-1} and azaperon 4 mg kg^{-1} i.m. for sedation. After arrival in the laboratory, anaesthesia was induced with pentobarbitone 15–20 mg kg^{-1} administered intravenously (i.v.). During the study period, the animals were carefully monitored and checked 24 h/day for 5 days. They were sedated using isoflurane inhalation (Abbott Laboratories, 0.8–1.3% end-tidal concentration) supplemented by intravenous bolus doses of morphine and ketamine as needed. After induction, the trachea was cannulated via a midline incision, and a tracheostomy tube was placed distally to the cricothyroid cartilage. All animals were then mechanically normoventilated using volume-controlled ventilation (Servo 300; Siemens Elema) at a FiO_2 of 0.21–0.30 and a respiratory rate of 20 breaths per minute. During the whole study period, the inspired tidal volume was adjusted to 12–15 ml kg^{-1} to maintain arterial oxygen and carbon dioxide tensions within normal limits. Arterial and central venous catheters were placed in the common carotid artery and the internal jugular vein via a separate incision on the neck and under antiseptic conditions. Arterial and central venous blood pressures, airway pressures, rectal temperature and standard three-lead ECG were continuously monitored. Excessive heat loss was avoided by covering the animals when necessary with a warming blanket. A urinary catheter was placed in the urinary bladder, through a low midline abdominal incision, for continuous monitoring of urinary output. Arterial acid–base balance, oxygen and carbon dioxide tensions, electrolytes and blood glucose concentration were monitored using ABL 2 and OSM2 analyzers (Radiometer) and a Glucometer (Bayer Health Care). A continuous infusion of Ringer's acetate at 2,000–4,000 ml day^{-1} was given for fluid replacement and to assure a urinary output of 25–50 ml h^{-1} throughout the study period. All wounds were carefully cleaned and closed by standard sutures to avoid unwanted contamination.

The animals were randomised into (a) a sham-operated control (CTL) group ($N=4$) where mechanical ventilation and sedation was applied for 3 h and (b) a mechanically ventilated (MV) experimental group ($N=4$) where ventilation was effective for 5 days and accompanied by continuous sedation as described above. In the MV group, animal position was altered daily between the left and right lateral position to improve gas exchange, allow pulmonary secretion to be removed via tracheal suctioning and to avoid pressure wounds. In addition, prophylactic antibiotic (Streptocillin Vet; Boehringer) was administered i.v. once daily. During the 5-day period, nutritional support was provided by a continuous infusion of buffered glucose 2.5 mg ml^{-1} , and the infusion rate was adjusted to maintain a tight control of arterial blood glucose levels at 4–8 mM. No vasopressors or additional fluids were given to any of the animals.

The study protocol was approved by the Local Ethical Committee on Animal Research at Linköping University (Dnr 15/97).

Muscle biopsies and permeabilisation of fibres

Biopsy specimens (2×2 cm) from the left anterior costal diaphragm were obtained after abdominal incision prior to euthanising the animal at the end point of the experiment (day 5) for all piglets from the MV group. In the CTL group, piglets were killed at the start point of the experiment (3 h). Biopsy specimen were placed in relaxing solution at 4°C, and bundles of ~50 fibres were dissected free and then tied with surgical silk to glass capillary tubes at slightly stretched lengths. The muscle bundles were then treated with skinning solution (relaxing solution containing glycerol; 50:50 v/v) for 24 h at 4°C, after which they were transferred to -20°C. The muscle bundles were treated with sucrose, a cryo-protectant, within 1–2 weeks for long-term storage [7]. After the sucrose treatment, muscle bundles were detached from the capillary tubes and snap frozen in liquid nitrogen-chilled propane and stored at -160°C.

Single muscle fibre experimental procedure

On the day of an experiment, a fibre segment 1 to 2 mm long was left exposed to the experimental solution between connectors leading to a force transducer (model 400A, Aurora Scientific) and a lever arm system (model 308B, Aurora Scientific) [22]. The total compliance of the attachment system was carefully checked and remained similar for all the single muscle fibres tested (5±0.5% of the fibre length). The apparatus was mounted on the stage of an inverted microscope (model IX70; Olympus). While the fibre segments were in relaxing solution, the sarcomere length was set to 2.65–2.75 μm (optimal sarcomere length for cells from these animals) by adjusting the overall segment length [15]. The diameter of the fibre segment between the connectors was measured through the microscope at a magnification of ×320 with an image analysis system prior to the mechanical experiments. Fibre depth was measured by recording the vertical displacement of the microscope nosepiece while focusing on the top and bottom surfaces of the fibre. The focusing control of the microscope was used as a micrometre. Fibre CSA was calculated from the diameter and depth, assuming an elliptical circumference, and was corrected for the 20% swelling that is known to occur during skinning [22].

Relaxing and activating solutions contained (in mM) 4 Mg-ATP, 1 free Mg²⁺, 20 imidazole, 7 EGTA, 14.5 creatine phosphate, and KCl to adjust the ionic strength to 180 mM. The pH was adjusted to 7.0. The concentrations of free Ca²⁺ were 10⁻⁹M (relaxing solution) and 10^{-6.2},

10^{-6.0}, 10^{-5.8}, 10^{-5.5}, 10^{-5.2}, 10^{-5.0} and 10^{-4.5}M (activating solutions), expressed as pCas (i.e. -log [Ca²⁺]). Apparent stability constants for Ca²⁺-EGTA were corrected for temperature (15°C) and ionic strength (180 mM). The computer programme of Fabiato [6] was used to calculate the concentrations of each metal, ligand and metal–ligand complex. EMD 57033 was a gift from Dr N. Beier (E. Merck Pharmaceuticals). It was prepared as a 10 mM stock solution in DMSO and was diluted in relaxing solution to obtain a final EMD 57033 concentration of 30 μM and a DMSO concentration of 0.3% (v/v). Control solutions were prepared with an equivalent volume of DMSO, which had no effect on the fibres.

At 15°C, immediately preceding each activation, the fibre was immersed for 10–20 s in a solution with a reduced Ca²⁺-EGTA buffering capacity. This solution is identical to the relaxing solution except that the EGTA concentration is reduced to 0.5 mM, which results in more rapid attainment of steady-state force during subsequent activation.

Force This was calculated as the difference between the steady-state isometric force in activating solutions and the resting force measured in the same segment while in the relaxing solution. Force was adjusted for CSA.

Stiffness Once steady-state isometric force was reached, small-amplitude sinusoidal changes in length (ΔL±0.2% of fibre length) were applied at 500 Hz at one end of the fibre [20]. The resultant force response (ΔF) was measured, and the mean of 20 consecutive readings of ΔL and ΔF was used to determine stiffness. The actual elastic modulus (E) was calculated as the difference between E in activating solutions and resting E measured in the same segment in the relaxing solution. E was determined as follows [21]:

$$E = (\Delta F / \Delta L) \times (\text{fiber length} / \text{CSA})$$

Relative force–pCa and stiffness–pCa relationships Each fibre was exposed to different solutions with pCas varying from 9.0 to 4.5. Force and stiffness were normalised to maximum force and stiffness at pCa 4.5, allowing the construction of relative force–pCa and relative stiffness–pCa curves. To determine the midpoint (termed pCa₅₀) and the Hill coefficient (nH) from the pCa curves, data were fitted (SigmaPlot 5.0 and Origin 6.1 Professional software; Jandel Scientific) using a three-parameter Hill equation:

$$X = [\text{Ca}^{2+}]^{\text{nH}} / \{ [\text{Ca}_{50}]^{\text{nH}} + [\text{Ca}^{2+}]^{\text{nH}} \}$$

Where X is the relative force or relative stiffness, -log [Ca₅₀] is the midpoint (pCa₅₀), and nH is the Hill coefficient.

For contractile measurements, strict acceptance criteria were applied. First, the sarcomere length was checked during the experiments, using a high-speed video analysis system (model 901A HVSL, Aurora Scientific). A muscle fibre was accepted and included in the analyses: (a) if the sarcomere length of a single muscle fibre changed $<0.10 \mu\text{m}$ between relaxation and maximum activation and (b) if maximal force changed $<10\%$ from first to final activation [22].

After the mechanical measurements, each fibre was placed in urea buffer (120 g urea, 38 g thiourea, 70 ml H_2O , 25 g mixed bed resin, 2.89 g dithiothreitol, 1.51 g Trizma base, 7.5 g SDS, and 0.004% bromophenol blue) in a plastic microcentrifuge tube and stored at -80°C .

MyHC isoform expression

The MyHC isoform composition of fibres was determined by 6% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Sample loads were kept small (equivalent to $\sim 0.05 \text{ mm}$ of fibre segment) to improve the resolution of the MyHC bands. Electrophoresis was performed at 120 V for 24 h with a Tris–glycine electrode buffer (pH 8.3) at 15°C (SE 600 vertical slab gel unit, Hoefer Scientific Instruments). The gels were silver-stained and subsequently scanned in a soft laser densitometer (Molecular Dynamics) with a high spatial resolution ($50 \mu\text{m}$ pixel spacing) and 4,096 optical density levels. Due to the small number of fibres expressing the type IIX MyHC isoform, comparisons were restricted to fibres expressing the type I and IIA MyHC isoforms.

MyHC, MyLC and actin quantification MyHC, myosin light chain (MyLC)1s/f, MyLC2s/f and actin contents of single fibres were determined by 12% SDS-PAGE (Fig. 1). The acrylamide concentration was 4% (wt/vol) in the stacking gel and 12% in the running gel, and the gel matrix included 10% glycerol. The gels were stained with Coomassie blue (0.5 g brilliant blue, 225 ml MeOH, 225 ml distilled H_2O , and 50 ml acetic acid). The relative contents were then calculated from the densitometric scanning (see above).

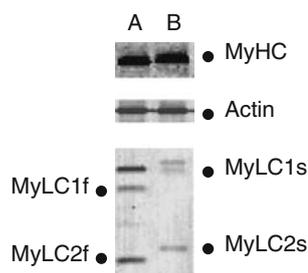


Fig. 1 Typical electrophoretograms showing MyHC, actin, MyLC1 and MyLC2 expressed in type IIA (A) and I fibres (B)

Statistical analysis

A total of 127 diaphragm fibres obtained from four CTL piglets and four MV piglets were used, i.e. a minimum of 15 acceptable fibres per animal. In the present manuscript, data are presented as means \pm standard error of the means (SEMs). Sigma Stat software (Jandel Scientific) was used to generate descriptive statistics. A three-way ANOVA (group[CTL–MV] \times fibre type[I – IIA] \times compound treatment[before–after]) was applied for each parameter (P_0/CSA , E_0 , $p\text{Ca}_{50}$ of force, nH of force, $p\text{Ca}_{50}$ of stiffness and nH of stiffness). In addition, a two-way ANOVA (group[CTL – MV] \times fibre type[I – IIA]) was performed for each proteins ratio. In case of differences, the post hoc Tukey test was performed. The level of significance was set at $p < 0.05$.

Results

After permeabilisation, muscle fibres were isolated from the bundles and mounted for contractile analyses. A total of 127 muscle fibres expressing the type I and IIA MyHC isoforms from the MV and CTL groups fulfilled the criteria for acceptance and were included in the analyses.

VIDD pathophysiology

At $p\text{Ca}$ 4.5, force normalised to cross-sectional area, i.e. specific force (P_0/CSA), and stiffness (E_0) were lower in both types I and IIA fibres from the MV group than the CTL animals (Table 1). It should be noticed that $p\text{Ca}$ 4.5 corresponded to maximum activating Ca^{2+} concentration. In fact, previous data have shown no significant difference between $p\text{Ca}$ 4.0 and 4.5.

The dependences of force and stiffness on Ca^{2+} concentration were characterised by two parameters obtained from fitting the data to the Hill equation (see “Materials and methods”), i.e. the $p\text{Ca}$ required to elicit half of the maximal force production ($p\text{Ca}_{50}$ or Ca^{2+} sensitivity) and the steepness (nH or Hill coefficient; Fig. 2). $p\text{Ca}_{50}$ of force was lower for type I and IIA fibres, whereas nH of force was higher solely in type I fibres from MV piglets when comparing with CTL animals (Table 2). $p\text{Ca}_{50}$ of stiffness was also lower for type I and IIA fibres and nH higher in fibres expressing the slow MyHC isoform in MV animals compared with CTL piglets (Table 2). Stiffness depends on the number of attached cross-bridges [10], the force/compliance of each cross-bridge and the compliance of the structures in series with the cross-bridges (the thick filament, proteins along the thin filament and probably also Z-disks) [8, 9, 32]. The strong linear relationships between the relative stiffness and the relative force in both mechanically ventilated and control piglets (Fig. 2) suggest

Table 1 Force and stiffness at maximal Ca²⁺ concentration

EMD 57033	Sham-operated				Mechanically ventilated			
	Type I		Type IIa		Type I		Type IIa	
	Treated	Control	Treated	Control	Treated	Control	Treated	Control
Specific force (N cm ⁻²)	11.90±0.90 (n=36)	14.50±1.40 (n=28)	13.90±1.10 (n=31)	13.80±1.40 (n=21)	7.80±0.40 ^a (n=30)	7.70±0.80 ^a (n=24)	8.90±0.50 (n=30)	7.90±0.80 (n=18)
Stiffness (N cm ⁻²)	1,370±110 (n=36)	1,400±150 (n=28)	1,420±190 (n=31)	1,460±210 (n=21)	650±60 ^a (n=36)	800±60 ^a (n=24)	710±60 (n=30)	840±80 (n=18)

All values are means±SEMs

^aSignificantly different from CTL (sham-operated)

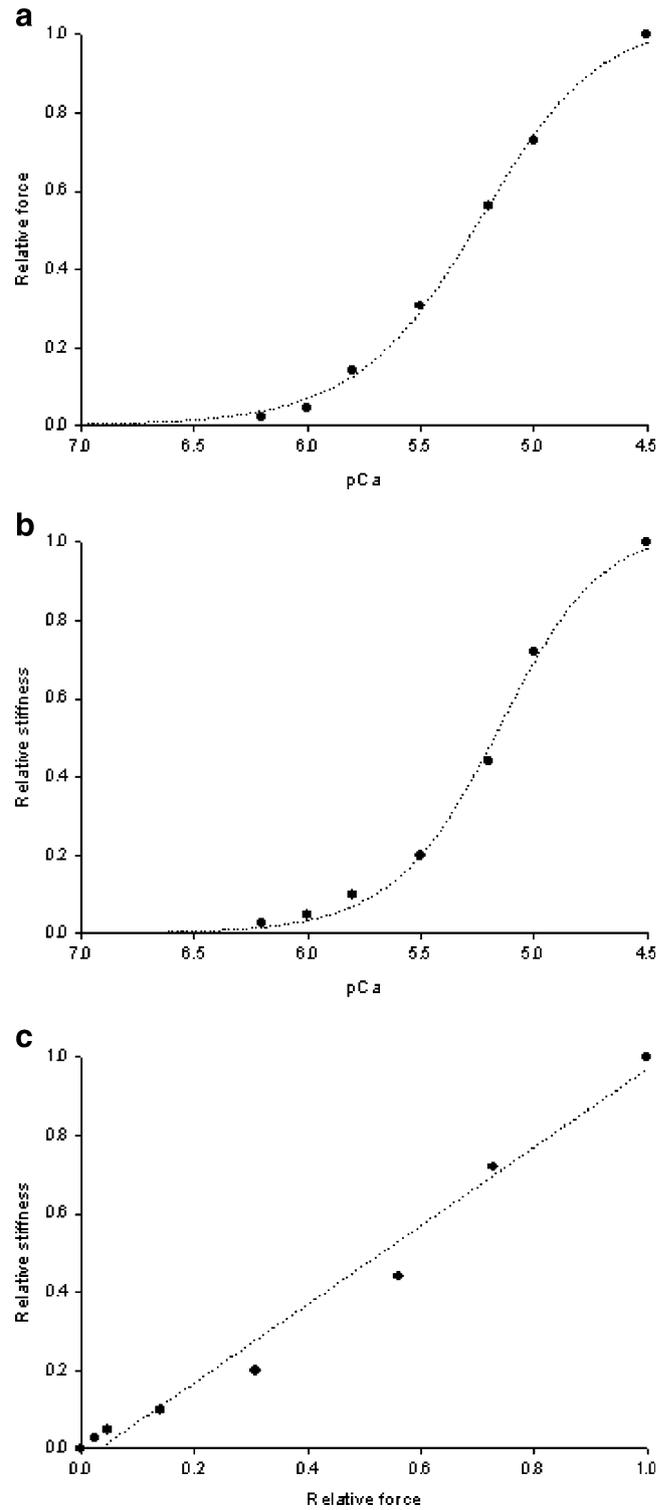


Fig. 2 Typical relative force–pCa (a), relative stiffness (b) and relative stiffness–relative force (c) curves in a type I fibre from a piglet belonging to the CTL group

Table 2 Changes in Ca^{2+} activation of force

EMD 57033	Sham-operated				Mechanically ventilated			
	Type I		Type IIa		Type I		Type IIa	
	–	Treated	–	Treated	–	Treated	–	Treated
$p\text{Ca}_{50}$ of force	5.21 ± 0.02 ($n=36$)	5.33 ± 0.03^a ($n=28$)	5.19 ± 0.04 ($n=31$)	5.28 ± 0.03^a ($n=21$)	4.98 ± 0.02^b ($n=36$)	5.08 ± 0.03^a ($n=30$)	5.08 ± 0.02^b ($n=24$)	5.13 ± 0.04 ($n=18$)
nH of force	1.97 ± 0.16 ($n=36$)	2.33 ± 0.21 ($n=28$)	2.12 ± 0.09 ($n=31$)	2.06 ± 0.15 ($n=21$)	3.15 ± 0.37^b ($n=36$)	2.59 ± 0.29 ($n=30$)	2.45 ± 0.29 ($n=24$)	2.14 ± 0.13 ($n=18$)
$p\text{Ca}_{50}$ of stiffness	5.19 ± 0.02 ($n=36$)	5.35 ± 0.02^a ($n=28$)	5.22 ± 0.03 ($n=31$)	5.35 ± 0.06^a ($n=21$)	4.96 ± 0.02^b ($n=36$)	5.10 ± 0.03^a ($n=30$)	5.10 ± 0.03^b ($n=24$)	5.17 ± 0.05 ($n=18$)
nH of stiffness	2.53 ± 0.23 ($n=36$)	2.77 ± 0.49 ($n=28$)	2.98 ± 0.32 ($n=31$)	2.31 ± 0.45 ($n=21$)	3.40 ± 0.26^b ($n=36$)	2.74 ± 0.32 ($n=30$)	2.48 ± 0.36 ($n=24$)	2.52 ± 0.27 ($n=18$)

All values are means \pm SEMs

(–) non-treated

^a Significantly different from before exposure to EMD 57033^b Significantly different from CTL (sham-operated)

that stiffness is primarily dependent on the number of attached cross-bridges at all levels of Ca^{2+} activation [30].

Otherwise, the MyHC, MyLC1s, MyLC1f, MyLC2s and MyLC2f to actin content ratios were unchanged among fibres expressing the type I and IIa MyHC isoforms (Table 3).

EMD 57033-related changes

At $p\text{Ca}$ 4.5, P_0/CSA and E_0 were not affected by the exposure to EMD 57033 in both types I and IIa fibres from the MV nor CTL groups (Table 1).

$p\text{Ca}_{50}$ of force, on the other hand, increased in type I fibres from MV piglets as well as in type I and IIa fibres from CTL animals (Table 2). The Ca^{2+} sensitizer did not significantly affect nH of force (Table 2), and it provoked an increase in $p\text{Ca}_{50}$ of stiffness in type I fibres from piglets belonging to MV group and in type I and IIa fibres from CTL animals whereas nH remained unchanged (Table 2). The maintained linear relationships (relative stiffness vs. relative force) in mechanically ventilated and control piglets after exposure to EMD 57033 suggest that stiffness remains primarily dependent on the number of attached cross-bridges at all levels of Ca^{2+} activation also after the treatment with the Ca^{2+} sensitizer.

Discussion

The results from the animal model emphasise that VID D pathophysiology in critically ill patients is complex; it is characterised not solely by atrophy and decreased force-generating capacity (specific force) but also by a reduction in the Ca^{2+} sensitivity of contraction. Results from the present study also demonstrate that some of these modifications are not immutable and can, in part, be counterbalanced, as attested by the increased Ca^{2+} sensitivities of force and stiffness in fibres exposed to EMD 57033. Under certain conditions, Ca^{2+} sensitisation may offer a promising mechanism to decrease VID D and weaning problems.

VID D pathophysiology

In addition to a dramatic decrease in specific force (Table 1) due to a loss in the total number of strongly attached cross-bridges (Table 1), a significant desensitisation of force production to Ca^{2+} concentration was observed as indicated by the rightward shift of $p\text{Ca}_{50}$ of force in type I and IIa fibres from piglets that were mechanically ventilated for 5 days compared with control animals, i.e. a higher Ca^{2+} concentration was required to activate the diaphragm muscle cells. This indicates a less efficient regulation of

Table 3 Myosin and actin quantifications

	Sham-operated		Mechanically ventilated	
	Type I	Type IIa	Type I	Type IIa
MyHC to actin ratio	1.55±0.18 (n=21)	1.51±0.23 (n=25)	1.25±0.17 (n=30)	1.36±0.16 (n=20)
MyLC1f to actin ratio	0.02±0.02 (n=21)	0.08±0.02 (n=25)	0.02±0.03 (n=30)	0.10±0.03 (n=20)
MyLC1s to actin ratio	0.07±0.02 (n=21)	0.06±0.02 (n=25)	0.09±0.03 (n=30)	0.08±0.05 (n=20)
MyLC2f to actin ratio	0.04±0.01 (n=21)	0.08±0.02 (n=25)	0.03±0.02 (n=30)	0.10±0.03 (n=20)
MyLC2s to actin ratio	0.11±0.04 (n=21)	0.02±0.03 (n=25)	0.12±0.04 (n=30)	0.02±0.02 (n=20)

All values are means ± SEMs

muscle contraction, since Ca^{2+} sequestration is an energy-consuming process [1]. To investigate whether this striking VIDD feature was due to a change in the number of strongly attached cross-bridges, the relative stiffness– $p\text{Ca}$ relationship was studied. A lower $p\text{Ca}_{50}$ of stiffness was observed in type I and IIa fibres from mechanically ventilated piglets than controls (Table 2), indicating a decrease in the relative number of recruited cross-bridges at each sub-maximal Ca^{2+} concentration. We have previously observed a similar desensitisation to Ca^{2+} concentration in limb muscle fibres with a preferential loss of myosin in patients with acute quadriplegic myopathy [26]. However, in the present study, the MyHC, MyLC1s, MyLC1f, MyLC2s and MyLC2f to actin content ratios remained unchanged. Hence, the underlying mechanisms in VIDD remain undefined and may arise from other factors than altered contents of, e.g. myosin and actin. This is consistent with the slow turnover rates of these proteins with half-lives of 5 days or longer [19, 42]. Post-translational modifications, including oxidation [43], on the other hand, offer alternative mechanisms underlying the modified propagation of the Ca^{2+} signal from the thin filament proteins to the cross-bridges in VIDD. Skeletal muscle is particularly vulnerable to oxidative stress. Six hours of mechanical ventilation promotes oxidative injury in the diaphragm, as indicated by the large increase in carbonyl derivatives [43]. It notably results in oxidation of myofibrillar proteins at molecular masses of 200 (MyHC) and 40 kDa (actin), altering their function [43].

In VIDD, other thin filament proteins may also be affected as indicated by the greater Hill coefficient (nH) of force and stiffness in type I fibres from piglets that were mechanically ventilated for 5 days compared with control animals. That is, the increase in nH and decrease in $p\text{Ca}_{50}$ may reflect a shift towards a faster troponin subunit isoform. The half-life of these particular proteins is approximately 4 days [19, 42]; a slow-to-fast isoform transformation cannot be excluded as previously shown during unloading [23]. In the present study, despite the use of sensitive silver-stained SDS-PAGE gels, we were not

able to clearly identify and quantify the different troponin isoforms in single cells.

It appears unlikely that the anaesthetic agents used in the present experiment enhance the muscle fibre dysfunction considering that isoflurane and other intravenous anaesthetics such as ketamine, midazolam, propofol and thiopental have no direct effects on diaphragm contractility [2, 24].

EMD 57033-related changes

Seeking therapeutic strategies to minimise VIDD and Ca^{2+} desensitisation is clinically relevant. Ca^{2+} sensitisers, including EMD 57033, have been developed in attempts to solve similar problems in cardiac muscle [3, 12]. EMD 57033 is a prototype Ca^{2+} sensitiser. Based on solution studies, its main mechanism of action consists of Ca^{2+} sensitisation of some of the contractile proteins, i.e. thin filament proteins. This is mediated through Ca^{2+} -dependent binding of the pharmacological agent to the Ca^{2+} subunit of the troponin complex, troponin C, more precisely to its slow skeletal/cardiac isoform, stabilising the Ca^{2+} -induced conformation of the contractile proteins. EMD 57033 binds directly to the C-terminal region of troponin C, thereby affecting troponin C interaction with the inhibitory subunit of the troponin complex, troponin I [17, 41]. This displaces troponin I from actin towards troponin C, inducing the first tropomyosin movement towards the inner domain of actin. This will initiate an improved cross-bridge recruitment, thereby changing cross-bridge transition rates [13, 18] and ultimately resulting in an increased force-generating capacity.

The leftward shift of $p\text{Ca}_{50}$ of force in type I fibres from mechanically ventilated and control piglets exposed to the EMD 57033 compound (Table 2) indicates a significant hypersensitisation of force production at each sub-maximal Ca^{2+} concentration, i.e. less Ca^{2+} was needed to activate the diaphragm muscle fibres. To evaluate whether this effect was caused by an alteration in the number of strongly attached cross-bridges, the

relative stiffness– pCa relationship was assessed. The higher pCa_{50} of stiffness in type I fibres from piglets belonging to MV and CTL groups following exposure to EMD 57033 (Table 2) demonstrates an enhanced relative number of recruited cross-bridges at the different Ca^{2+} concentrations. In spite of the unspecific inotropic effect of EMD 57033 on type I fibres from both mechanically ventilated and control piglets, it may nevertheless have a beneficial impact on diaphragm muscle function in VIDD. Similar beneficial action of another Ca^{2+} sensitizer, i.e. levosimendan, was recently reported in diaphragm muscle cells expressing the type I MyHC isoform from patients with chronic obstructive pulmonary disease [37].

The enhanced Ca^{2+} activation of contraction induced by EMD 57033 in type IIa fibres from control animals was an unexpected finding, especially since EMD 57033 has not been shown to bind to the fast skeletal troponin C isoform. However, the co-expression of slow skeletal/cardiac and fast skeletal troponin C isoforms [23] may offer an explanation to this result. The difference between type IIa control fibres and cells from mechanically ventilated animals may be secondary to a slow-to-fast troponin C isoform transition, i.e. a decrease in the expression of slow skeletal/cardiac troponin C isoform in favour of fast troponin C isoform in type IIa fibres, during mechanical ventilation as previously shown during unloading [23].

Clinical repercussions

Even though weaning failure in critically ill mechanically ventilated patients arises from multiple factors, including residual effects of sedative agents (e.g. hypnotics in combination with analgetics), abnormalities in lung mechanics, impaired oxygen delivery to muscles and psychological problems, VIDD appears to play a key role [11]. EMD 57033 partially counterbalances VIDD by restoring the force-generating capacity, especially at sub-maximal Ca^{2+} concentrations. Under in vivo conditions, diaphragm muscle does not generate maximum force, but shortens against sub-maximal loads, and the observed in vitro effects of EMD 57033 present significant potential therapeutic advantages [3, 12] such as (a) improvement of the effectiveness without increasing energy consumption during basal metabolism, excitation–contraction coupling and force generation and (b) no change in intracellular Ca^{2+} concentration, reducing the risk of Ca^{2+} overload.

Conclusions

Alterations in Ca^{2+} sensitivities of force and stiffness were observed in diaphragm muscle fibres from mechanically ventilated piglets compared with sham-operated animals, suggesting a less efficient Ca^{2+} activation of cross-bridges

and subsequent force production. To further assess whether the impaired Ca^{2+} activation is reversible, the fibres were exposed to a Ca^{2+} sensitizer. The present results show that the addition of EMD 57033 partially restored the Ca^{2+} activation, indicating that treatment with Ca^{2+} sensitizers may provide an efficient therapeutic strategy in reducing VIDD.

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References

- Barclay CJ, Woledge RC, Curtin NA (2007) Energy turnover for Ca^{2+} cycling in skeletal muscle. *J Muscle Res Cell Motil* 28:259–274
- Bouhemad B, Langeron O, Orliaguet G, Coriat P, Riou B (2002) Effects of halothane and isoflurane on the contraction, relaxation and energetics of rat diaphragmatic muscle. *Br J Anaesth* 89:479–485
- Endoh M (2008) Cardiac Ca^{2+} signaling and Ca^{2+} sensitizers. *Circ J* 72:1915–1925
- Esteban A, Anzueto A, Alia I, Gordo F, Apezteguia C, Palizas F, Cide D, Goldwaser R, Soto L, Bugedo G, Rodrigo C, Pimentel J, Raimondi G, Tobin MJ (2000) How is mechanical ventilation employed in the intensive care unit? An international utilization review. *Am J Respir Crit Care Med* 161:1450–1458
- Esteban A, Frutos F, Tobin MJ, Alia I, Solsona JF, Valverde I, Fernandez R, de la Cal MA, Benito S, Tomas R et al (1995) A comparison of four methods of weaning patients from mechanical ventilation. Spanish Lung Failure Collaborative Group. *N Engl J Med* 332:345–350
- Fabiato A (1988) Computer programs for calculating total from specified free or free from specified total ionic concentrations in aqueous solutions containing multiple metals and ligands. *Methods Enzymol* 157:378–417
- Frontera WR, Larsson L (1997) Contractile studies of single human skeletal muscle fibers: a comparison of different muscles, permeabilization procedures, and storage techniques. *Muscle Nerve* 20:948–952
- Galler S, Hilber K (1998) Tension/stiffness ratio of skinned rat skeletal muscle fibre types at various temperatures. *Acta Physiol Scand* 162:119–126
- Higuchi H, Yanagida T, Goldman YE (1995) Compliance of thin filaments in skinned fibers of rabbit skeletal muscle. *Biophys J* 69:1000–1010
- Huxley AF (1971) The activation of striated muscle and its mechanical response. *Proc R Soc Lond B Biol Sci* 178:1–27
- Jubran A (2006) Critical illness and mechanical ventilation: effects on the diaphragm. *Respir Care* 51:1054–1061 discussion 1062–1054
- Kass DA, Solaro RJ (2006) Mechanisms and use of calcium-sensitizing agents in the failing heart. *Circulation* 113:305–315
- Kraft T, Brenner B (1997) Force enhancement without changes in cross-bridge turnover kinetics: the effect of EMD 57033. *Biophys J* 72:272–281

14. Lamb GD, Posterino GS (2003) Effects of oxidation and reduction on contractile function in skeletal muscle fibres of the rat. *J Physiol* 546:149–163
15. Larsson L, Moss RL (1993) Maximum velocity of shortening in relation to myosin isoform composition in single fibres from human skeletal muscles. *J Physiol* 472:595–614
16. Levine S, Nguyen T, Taylor N, Friscia ME, Budak MT, Rothenberg P, Zhu J, Sachdeva R, Sonnad S, Kaiser LR, Rubinstein NA, Powers SK, Shrager JB (2008) Rapid disuse atrophy of diaphragm fibers in mechanically ventilated humans. *N Engl J Med* 358:1327–1335
17. Li MX, Spyrapoulos L, Beier N, Putkey JA, Sykes BD (2000) Interaction of cardiac troponin C with Ca²⁺ sensitizer EMD 57033 and cardiac troponin I inhibitory peptide. *Biochemistry* 39:8782–8790
18. Lipscomb S, Preston LC, Robinson P, Redwood CS, Mulligan IP, Ashley CC (2005) Effects of troponin C isoform on the action of the cardiotoxic agent EMD 57033. *Biochem J* 388:905–912
19. Martin AF (1981) Turnover of cardiac troponin subunits. Kinetic evidence for a precursor pool of troponin-I. *J Biol Chem* 256:964–968
20. Martyn DA, Smith L, Kreutziger KL, Xu S, Yu LC, Regnier M (2007) The effects of force inhibition by sodium vanadate on cross-bridge binding, force redevelopment, and Ca²⁺ activation in cardiac muscle. *Biophys J* 92:4379–4390
21. McDonald KS, Fitts RH (1995) Effect of hindlimb unloading on rat soleus fiber force, stiffness, and calcium sensitivity. *J Appl Physiol* 79:1796–1802
22. Moss RL (1979) Sarcomere length-tension relations of frog skinned muscle fibres during calcium activation at short lengths. *J Physiol* 292:177–192
23. Mounier Y, Tiffreau V, Montel V, Bastide B, Stevens L (2009) Phenotypical transitions and Ca²⁺ activation properties in human muscle fibers: effects of a 60-day bed rest and countermeasures. *J Appl Physiol* 106:1086–1099
24. Nishina K, Mikawa K, Kodama S, Kagawa T, Uesugi T, Obara H (2003) The effects of enflurane, isoflurane, and intravenous anesthetics on rat diaphragmatic function and fatigability. *Anesth Analg* 96:1674–1678 table of contents
25. Norman H, Kandala K, Kolluri R, Zackrisson H, Nordquist J, Walther S, Eriksson LI, Larsson L (2006) A porcine model of acute quadriplegic myopathy: a feasibility study. *Acta Anaesthesiol Scand* 50:1058–1067
26. Ochala J, Larsson L (2008) Effects of a preferential myosin loss on Ca²⁺ activation of force generation in single human skeletal muscle fibres. *Exp Physiol* 93:486–495
27. Ochala J, Li M, Ohlsson M, Oldfors A, Larsson L (2008) Defective regulation of contractile function in muscle fibres carrying an E41K beta-tropomyosin mutation. *J Physiol* 586:2993–3004
28. Powers SK, Kavazis AN, McClung JM (2007) Oxidative stress and disuse muscle atrophy. *J Appl Physiol* 102:2389–2397
29. Powers SK, Shanely RA, Coombes JS, Koesterer TJ, McKenzie M, Van Gammeren D, Cicale M, Dodd SL (2002) Mechanical ventilation results in progressive contractile dysfunction in the diaphragm. *J Appl Physiol* 92:1851–1858
30. Regnier M, Martin H, Barsotti RJ, Rivera AJ, Martyn DA, Clemmens E (2004) Cross-bridge versus thin filament contributions to the level and rate of force development in cardiac muscle. *Biophys J* 87:1815–1824
31. Sassoon CS, Zhu E, Caiozzo VJ (2004) Assist-control mechanical ventilation attenuates ventilator-induced diaphragmatic dysfunction. *Am J Respir Crit Care Med* 170:626–632
32. Seow CY, Shroff SG, Ford LE (1997) Detachment of low-force bridges contributes to the rapid tension transients of skinned rabbit skeletal muscle fibres. *J Physiol* 501(Pt 1):149–164
33. Shanely RA, Van Gammeren D, Deruisseau KC, Zergeroglu AM, McKenzie MJ, Yarasheski KE, Powers SK (2004) Mechanical ventilation depresses protein synthesis in the rat diaphragm. *Am J Respir Crit Care Med* 170:994–999
34. Shanely RA, Zergeroglu MA, Lennon SL, Sugiura T, Yimlamai T, Enns D, Belcastro A, Powers SK (2002) Mechanical ventilation-induced diaphragmatic atrophy is associated with oxidative injury and increased proteolytic activity. *Am J Respir Crit Care Med* 166:1369–1374
35. Solaro RJ, Gambassi G, Warsaw DM, Keller MR, Spurgeon HA, Beier N, Lakatta EG (1993) Stereoselective actions of thiazolidinones on canine cardiac myocytes and myofilaments. *Circ Res* 73:981–990
36. Spencer T, Posterino GS (2009) Sequential effects of GSNO and H₂O₂ on the Ca²⁺ sensitivity of the contractile apparatus of fast- and slow-twitch skeletal muscle fibers from the rat. *Am J Physiol, Cell Physiol* 296:C1015–C1023
37. van Hees HW, Dekhuijzen PN, Heunks LM (2009) Levosimendan enhances force generation of diaphragm muscle from patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 179:41–47
38. Vannier C, Lakomkine V, Vassort G (1997) Tension response of the cardiotoxic agent (+)-EMD-57033 at the single cell level. *Am J Physiol* 272:C1586–C1593
39. Vassilakopoulos T, Petrof BJ (2004) Ventilator-induced diaphragmatic dysfunction. *Am J Respir Crit Care Med* 169:336–341
40. Vassilakopoulos T, Zakyntinos S, Roussos C (1996) Respiratory muscles and weaning failure. *Eur Respir J* 9:2383–2400
41. Wang X, Li MX, Spyrapoulos L, Beier N, Chandra M, Solaro RJ, Sykes BD (2001) Structure of the C-domain of human cardiac troponin C in complex with the Ca²⁺ sensitizing drug EMD 57033. *J Biol Chem* 276:25456–25466
42. Zak R, Martin AF, Blough R (1979) Assessment of protein turnover by use of radioisotopic tracers. *Physiol Rev* 59:407–447
43. Zergeroglu MA, McKenzie MJ, Shanely RA, Van Gammeren D, DeRuisseau KC, Powers SK (2003) Mechanical ventilation-induced oxidative stress in the diaphragm. *J Appl Physiol* 95:1116–1124