

Respiratory muscle contractile inactivity induced by mechanical ventilation in piglets leads to leaky ryanodine receptors and diaphragm weakness

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Abstract Respiratory muscle contractile inactivity during mechanical ventilation (MV) induces diaphragm muscle weakness, a condition referred to as ventilator-induced diaphragmatic dysfunction (VIDD). Although VIDD pathophysiological mechanisms are still not fully understood, it has been recently suggested that remodeling of the sarcoplasmic reticulum (SR) calcium release channel/ryanodine receptors (RyR1) in the diaphragm is a proximal mechanism of VIDD. Here, we used piglets, a large animal model of VIDD that is more relevant to human pathophysiology, to determine whether RyR1 alterations are observed in the presence of diaphragm weakness. In piglets, diaphragm weakness induced by 72 h of respiratory muscle unloading was associated with SR RyR1 remodeling and abnormal resting SR Ca²⁺ leak in the diaphragm. Specifically, following controlled mechanical ventilation, diaphragm contractile function was reduced. Moreover, RyR1 macromolecular complexes were more oxidized, S-nitrosylated and phosphorylated at Ser-2844 and depleted of the stabilizing subunit calstabin1 compared with controls on adaptive support ventilation that maintains diaphragmatic contractile activity. Our study strongly supports the hypothesis that RyR1 is a potential therapeutic target in VIDD and the interest of using small molecule drugs to prevent RyR1-mediated SR Ca²⁺ leak induced by respiratory muscle

unloading in patients who require controlled mechanical ventilation.

Keywords Mechanical ventilation · Diaphragm · Calcium homeostasis · Calcium channel

Introduction

Mechanical ventilation (MV) is the most efficient treatment for respiratory failure in intensive care units (ICU). However, the respiratory muscle contractile inactivity induced by MV (i.e., the unloading of the respiratory muscle) has various adverse cellular effects that lead, in a time-dependent way, to diaphragm weakness, atrophy and injury (Powers et al. 2009; Levine et al. 2008; Jaber et al. 2011), a pathological condition known as ventilator-induced diaphragmatic dysfunction (VIDD) (Vassilakopoulos and Petrof 2004). The identification of the different cellular pathways involved in diaphragm weakness is important because VIDD increases the difficulties of MV weaning (Tobin et al. 2009), with a major impact on healthcare costs (Esteban et al. 2008).

Oxidative stress is considered to be a major pathophysiological mechanism of VIDD (Shanely et al. 2002; Falk et al. 2006; Zergeroglu et al. 2003; Betters et al. 2004), and mitochondria are an essential source of reactive oxygen species (ROS) (Betters et al. 2004; Picard et al. 2012; Kavazis et al. 2009). It is not clear why mitochondrial ROS production is increased following respiratory muscle unloading induced by MV. However, this energetic homeostasis imbalance with acute substrate oversupply is a potential source of mitochondrial dysfunction (Picard et al. 2012).

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Recently, we showed that Ca^{2+} homeostasis impairment is involved in the structural and functional impairment of type 1 ryanodine receptors (RyR1) induced by oxidative stress (Matecki et al. 2016). RyRs are located in the sarcoplasmic reticulum (SR) and are responsible for the release of Ca^{2+} from this intracellular store during excitation–contraction (EC) coupling. RyR1-mediated Ca^{2+} release is modulated by regulatory proteins (for instance, the FK506 binding protein 1A, also known as calstabin1) that bind to its large cytoplasmic scaffold domain (Brillantes et al. 1994). Following protein kinase A (PKA)-mediated RyR1 phosphorylation and oxidation, the association between calstabin1 and the rest of the RyR1 complex is lost (Ward et al. 2003; Reiken et al. 2003), leading to leaky RyR1 and consequently to impaired Ca^{2+} handling and contractile dysfunction in many different conditions, such as VIDD (Matecki et al. 2016), heart failure (Ward et al. 2003), aging (2011), chronic muscle fatigue (Bellinger et al. 2008) and muscular dystrophy (Bellinger et al. 2009). This important cellular pathway could be considered as a proximal mechanism of the Ca^{2+} -dependent proteolysis and muscle atrophy observed in different animal models of VIDD (Shanely et al. 2002; Maes et al. 2007; Kavazis et al. 2007; McClung et al. 2009; Jung et al. 2010) and in human patients (Levine et al. 2008; Jaber et al. 2011). This suggests that RyR1 is a potential therapeutic target in VIDD.

We recently reported that in patients hospitalized in ICU and mechanically ventilated for a mean period of 4 days, post-translational RyR1 modifications in the diaphragm are a proximal mechanism of VIDD (Matecki et al. 2016). However, the mechanisms involved in such modifications remain unclear. Indeed, several causes could be involved, such as catecholamine stress, sepsis, mechanical stress induced by patient-ventilator asynchrony, drugs used for sedation or respiratory muscle unloading. Using a murine model of VIDD, we established the link between respiratory muscle unloading and diaphragm force deficit (Matecki et al. 2016). However, mouse models of VIDD with the simple device used for mechanical ventilation (a syringe that pumps a constant level of air in the airways) are physiologically not comparable to human patients. Moreover, this model does not allow monitoring the diaphragm activity to confirm the lack of contraction at each breathing cycle.

In view of future clinical trials, it is mandatory to establish the link between respiratory muscle unloading and RyR1 dysfunction in an animal model that is physiologically similar to humans. Indeed, respiratory muscle unloading is the only putative cause of RyR1 dysfunction that cannot be removed when MV is used to efficiently ventilate lungs. Thus, a larger animal model of VIDD, in which diaphragm contractile activity can be monitored during MV with devices similar to those used in patients admitted in

ICU, is required to determine the direct consequence of diaphragm contractile inactivity on RyR1 function. Using a piglet model of VIDD, we previously reported that total unloading of respiratory muscles during controlled mechanical ventilation (CMV) induces diaphragm weakness. This could be prevented by maintaining a certain level of diaphragmatic contractile activity using adaptive support ventilation (ASV) (Jung et al. 2010). Therefore, the aim of the present study was to evaluate in the same animal model, whether CMV for 3 days induced RyR1 remodeling and dysfunction. To specifically link RyR1 alteration to respiratory muscle unloading we tested whether RyR1 dysfunction could be prevented by ASV.

Methods

Piglet model

As previously described (Jung et al. 2010; Jaber et al. 2008), ten piglets (15–20 kg) were separated in two groups of five animals/each. In both groups, oxygenation was maintained with a fraction of inspired oxygen (FIO_2) ranging from 25 to 35%, tidal volume between 10 and 12 ml kg^{-1} body weight and breathing frequency from 15 to 30 cycles/min to maintain normocapnia. In the first group (MV group), piglets were mechanically ventilated using CMV, with a positive end-expiratory pressure level at 5 cm H_2O . The absence of spontaneous breathing was verified on the ventilator trend graphs, and the diaphragm electromyographic activity was measured to ensure the absence of diaphragm electrical activity. In the second group (control group), piglets were ventilated using ASV, with settings in phase with the piglet body weight (inspiratory flow trigger at 0.3 L min^{-1} , percentage of mechanical ventilation between 100 and 150%, positive end-expiratory pressure level at 5 cm H_2O , and expiratory trigger at 25% of the peak inspiratory flow). The diaphragm electromyographic activity was assessed to verify that piglets could breathe spontaneously. Both groups received the same care, except for the MV mode.

Briefly, piglets were anesthetized by intravenous injection of 5–6 mg kg^{-1} pentobarbital sodium, intubated with a cuffed endotracheal tube and mechanically ventilated (Galileo[®]; Hamilton Medical AG, Rhazuns, Switzerland). Anesthesia was maintained with continuous intravenous infusion of 15–20 mg kg^{-1} propofol, 0.1–0.3 mg kg^{-1} midazolam and 3–4 mg kg^{-1} h⁻¹ ketamine. The level of sedation was monitored by using a bispectral index system (BIS[®]; Aspect, Norwood, MA). Heating pads were employed, as needed, to maintain the normal body temperature of 38.5–39.5 °C. A carotid arterial catheter (PiCCO[®]; Pulsion, Munich, Germany) was inserted for monitoring

the heart rate, arterial blood pressure and cardiac output. The absence of spontaneous breathing in the MV group was verified using the ventilator trend graphs and the diaphragm electromyographic activity measurement.

Arterial carbon dioxide pressure was monitored using a capnograph (Deltatrac[®]; Datex-Ohmeda, Helsinki, Finland) and verified by arterial blood gas analysis (iSTAT[®]; Abbott, Abbott Park, IL). Parenteral nutrition was given from the first day (10% glucose, 20% amino acids solution and Hyperamine 20[®]; Braun, Boulogne Billancourt, France) to provide 30–35 kcal kg⁻¹ day⁻¹. All procedures were performed aseptically. All animals received prophylactic intravenous injections of antibiotics three times per day (100 mg kg⁻¹ day⁻¹ amoxicillin–clavulanate).

The diaphragm contractile function was assessed *in vivo* by measuring the transdiaphragmatic pressure (Pdi) (Jung et al. 2010). A double-lumen air-filled balloon-tipped catheter was placed transorally in the stomach and the distal third of the esophagus. Bipolar transvenous pacing catheters were introduced through each internal jugular vein and adjusted to stimulate the phrenic nerve and subsequently induce the diaphragm contraction by supramaximal stimulation at frequencies ranging from 20 to 120 Hz with trains of stimulation of 2 s and 150 ms.

RyR1 biochemical analysis

Diaphragm muscle biopsies were homogenized in 150 μ l of buffer containing 5% SDS, 5% beta-mercaptoethanol, 10% glycerol, 10mM EDTA and 50mM Tris/HCl buffer (pH 8.0). Each sample was immediately denatured at 90 °C for 4 min. After centrifugation (5000 rpm) at 4 °C, protein concentration in supernatants was measured in duplicate using the BCA protein assay. Samples were then diluted to the same concentration (2 μ g/ μ l) with loading buffer and aliquoted. RyR1 was immunoprecipitated from 250 μ g of protein homogenates using 4 μ g of home-made anti-RyR antibody (RyR1-1327, see below for description) in 0.5 ml of modified RIPA buffer (50mM Tris–HCl pH 7.4, 0.9% NaCl, 5.0 mM NaF, 1.0 mM Na₃VO₄, 1% Triton-X100, and protease inhibitors) at 4 °C for 1h. Immune complexes were incubated with protein A Sepharose beads (Amersham Pharmacia) at 4 °C for 1h and beads were washed three times with buffer. Proteins were separated on 4–20% SDS–PAGE gradient gels and transferred onto nitrocellulose membranes at 200 mA for 2 h (SemiDry transfer blot, Bio-Rad). To prevent non-specific antibody binding, membranes were incubated with blocking solution (LICOR Biosciences) and washed with Tris-buffered saline/0.1% Tween-20.

Blots were incubated with the primary anti-RyR1 antibody RyR1-1327. This is an affinity-purified rabbit polyclonal antibody that was raised against a KLH-conjugated

peptide with the amino acid sequence CAEPDITYEN-LRRS that corresponds to residues 1327–1339 of mouse skeletal RyR1 and an additional cysteine residue added to the amino terminus and then affinity-purified with the unconjugated peptide. Antibodies against calstabin1 (1:2500 in blocking buffer, LICOR Biosciences), human RyR2 phosphorylated on Ser-2808 (1:5000), PKA-phosphorylated mouse RyR1 (on Ser-2844), and S-nitrosylated cysteine residues (1:1000, Sigma) were also used. To determine RyR1 oxidation, immunoprecipitates were incubated with 2, 4-dinitrophenyl hydrazine and the derivatized carbonyls were detected using the OxyBlot Protein Oxidation Detection Kit (catalog S7150, Chemicon International Inc.). After three washes, membranes were incubated with infrared-labeled secondary antibodies. Samples from MV and control piglets were added on each gel and after normalization, RyR1 total levels were not different between groups.

Sarcoplasmic reticulum vesicle preparation

Piglet diaphragm muscle biopsies were homogenized on ice in 300 mM sucrose, 20 mM PIPES (pH 7.0) in the presence of protease inhibitors (Roche) and centrifuged at 8000 rpm (5900g) at 4 °C for 20 min. Supernatants were then ultracentrifuged at 32,000 rpm (100,000g) at 4 °C for 1 h. The final pellets that contain microsomal fractions enriched in SR vesicles were resuspended and aliquoted in 300 mM sucrose, 5 mM PIPES (pH 7.0) with protease inhibitors. Samples were frozen in liquid nitrogen and stored at –80 °C.

Single channel data from planar lipid bilayer measurements

Planar lipid bilayers were formed from a 3:1 mixture of phosphatidylethanolamine and phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) suspended (30 mg/ml) in decane by painting the lipid/decane solution across a 200- μ m aperture on one side of a polysulfonate cup (Warner Instruments) that separates two chambers. The *trans* chamber (1 ml), which represents the intra-SR (luminal) compartment, was connected to the headstage input of a bilayer voltage clamp amplifier (BC-525D, Warner Instruments) and the *cis* chamber (1 ml), which represents the cytoplasmic compartment, was held at virtual ground. Solutions in both chambers were as follows: 1mM EGTA, 250/125 mM HEPES/Tris, 50 mM KCl, 0.64 mM CaCl₂, pH 7.35 (*cis* solution), and 53 mM Ca(OH)₂, 50 mM KCl, 250mM HEPES, pH 7.35 (*trans* solution). The concentration of free Ca²⁺ in the *cis* chamber was calculated with the WinMaxC program (version 2.50; <http://www.stanford.edu/~cpatton/maxc.html>). SR vesicles were added to the *cis*

side and fusion with the lipid bilayer was induced by making the *cis* side hyperosmotic by addition of 400–500 mM KCl. After the appearance of potassium and chloride channels, the *cis* compartment was perfused with the *cis* solution. Single-channel currents were recorded at 0 mV using a Bilayer Clamp BC-535 amplifier (Warner Instruments), filtered at 1 kHz and digitized at 4 kHz. All experiments were performed at room temperature. Data acquisition was performed with the Digidata 1440A and Axoscope 10.2 software programs and recordings were analyzed using Clampfit 10.2 (Molecular Devices). The open probability (P_o) was calculated by 50% threshold analysis using a least 2 min of continuous recording, as previously described (Matecki et al. 2016, 2017; Bellinger et al. 2008). At the end of each experiment, 5 μ M ryanodine was added to the *cis* chamber to confirm that the channels were RyRs.

Statistical analysis

Data are presented as the mean \pm SEM. Differences between groups were analyzed with the ANOVA test. Statistical significance was set at $p < 0.05$.

Results

One limitation of using respiratory muscle biopsies from patient in ICU to investigate the pathophysiological mechanisms of VIDD is the potential influence of comorbid conditions and confounding factors associated with their critical condition. Therefore, to specifically determine the effect of respiratory muscle unloading on RyR1 properties and diaphragm function, we used diaphragm biopsies from healthy piglets that were anesthetized and ventilated using a controlled ventilation mode (MV group) or ASV (control group) for 72 h. In the MV group, as previously described (Jung et al. 2010), spontaneous diaphragm activity was observed for no more than 5% of all delivered breaths. Conversely, in the control group, 80% of breaths delivered by the ventilator were triggered by spontaneous contractile activity of the diaphragm. Throughout the study (3 days of CMV), animals were afebrile with normal intestinal transit and diuresis as well as normal fluid balance and stable hemodynamic parameters.

At the beginning of the ventilation (baseline), the maximal diaphragm strength, evaluated by measuring the maximal transdiaphragmatic pressure, was not significantly different in the MV and control group (49 ± 6 vs 43 ± 7 cm H₂O). In the control group (ASV mode), Pdi did not decrease at all tested frequencies between baseline and the end of the 3 days of ventilation (Fig. 1). This suggests that the ASV mode does not affect diaphragm function over 3 days. Conversely, in the MV group, Pdi was significantly

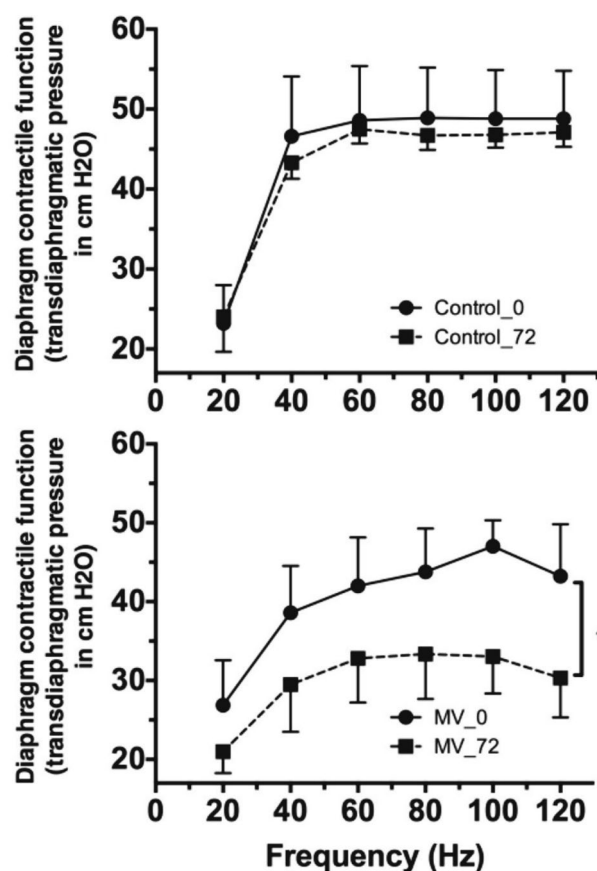


Fig. 1 Diaphragmatic contractile function was assessed by *in vivo* measurements of transdiaphragmatic pressure (Pdi) at $t=0$ (baseline) and $t=72$ (72 h of anesthesia) in control (control_0 and control_72) and mechanically ventilated piglets (MV_0 and MV_72), produced by supramaximal stimulation and expressed as a function of the stimulation frequency. MV_0 Piglets on controlled mechanical ventilation for less than 3 h. MV_72 Piglets on controlled mechanical ventilation for 3 days. Control_0 Piglets on assisted support ventilation for less than 3 h. Control_72 Piglets on assisted support ventilation during 3 days (* $p < 0.05$, MV_72 vs. MV_0)

lower after 3 days of MV compared with baseline (30 ± 5 versus 43 ± 6 cm H₂O, respectively; $p < 0.05$) (Fig. 1).

Then, to analyze the biochemical properties of the RyR1 macromolecular complex, SR fractions were purified from diaphragm muscle biopsies obtained after 72 h of ventilation in both groups. Western blot analysis after RyR1 immunoprecipitation showed that RyR1 S-nitrosylation, oxidation and phosphorylation on Ser-2844 as well as calstabin1 dissociation from RyR1 were significantly higher in MV samples compared with controls (ASV) (Fig. 2). This biochemical remodeling of RyR1 channels is known as the biochemical signature of “leaky” RyR1 channels (Bellinger et al. 2008) and is associated with a significant increase in RyR1 open probability (P_o) measured in channels incorporated in planar lipid bilayers. Accordingly, compared with non-remodeled RyR1 channels (control group) that

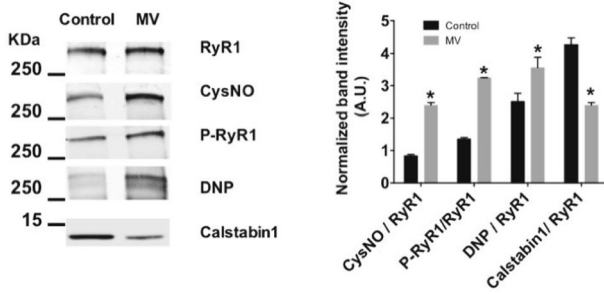


Fig. 2 Representative immunoblots of immunoprecipitated RyR1 from diaphragm muscle samples collected from control group (ventilated with ASV mode) and MV group (ventilated with CMV mode) after 72 h of ventilation (each blot corresponds to adjacent wells of the same gel) (left). Graph (right) shows the quantification relative to total RyR1 (mean ± SEM, n = 5 in both groups, *p < 0.05, MV vs. control). CysNO, thio-nitrosylation; DNP, 2,4-dinitrophenylhydrazine; P-RyR1, RyR1 phosphorylated at serine 2844. Control and MV refer to samples collected at t = 72. Data are expressed as the mean ± SEM

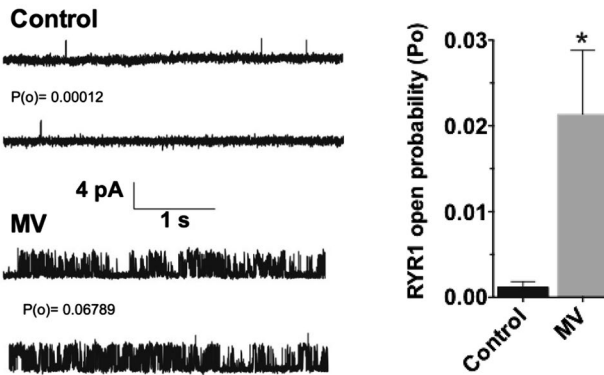


Fig. 3 Single channel traces of single RyR1 incorporated in planar lipid bilayers, corresponding to representative experiments performed with porcine diaphragm muscle biopsies from Control and MV animals after 72 h of ventilation. Respiratory muscle unloading increased RyR1 open probability (Po) value in MV group (n = 20 biopsies) compared with control group (n = 12 biopsies) (0.02130 ± 0.007519 and 0.00115 ± 0.00069, respectively; *p < 0.05 MV vs controls)

were tightly closed (Po = ~0) (Fig. 3), Po in the MV group increased to 0.021 ± 0.007 after 3 days of MV, suggesting increased SR Ca²⁺ leak.

Discussion

This study shows that, in piglets mechanically ventilated with CMV, for 72 h, the diaphragm exhibit the biochemical signature of leaky RyR1 channels, suggesting intracellular Ca²⁺ leak, as shown by the increased mean channel RyR1 open probability, associated with a reduced diaphragm

force production. By using piglets, an animal model physiologically similar to humans, and the same ventilation mode and anesthesia care routinely employed in ICU for patients with acute respiratory distress syndrome, we show here for the first time, that diaphragm inactivity, induced by CMV and associated with passive muscle fiber length changes, contributes to the RyR1 defect associated with diaphragm weakness. This demonstrates the link between respiratory muscle unloading after 3 days of CMV and RyR1 alteration associated with SR Ca²⁺ leak, which is considered to be the proximal mechanism of VIDD in ICU patients (Matecki et al. 2016).

Similar results were previously reported using mouse models of VIDD. Specifically, oxidative stress and catecholamine stress during MV have been recently involved in RyR1 dysfunction, and linked to diaphragm weakness in a murine model (Matecki et al. 2016). However, several limitations precluded the translation of this finding to the clinic, thus emphasizing the necessity to confirm, using a model closer to humans, that the RyR1 defect is linked to diaphragm unloading. Indeed, ventilators for mice are rudimentary, without any possibility to prove the lack of diaphragm contractile activity during mechanical ventilation. Moreover, in mice, the occurrence of asynchrony, defined as the mismatching between neural inspiratory and mechanical inspiratory time that induces episodic diaphragm contractions without air delivery, cannot be excluded (Sassoon and Foster 2001). As a consequence, the RyR1 defect observed in mechanically ventilated mice could have been caused mainly by the diaphragm mechanical stress caused by asynchrony, rather than unloading. Indeed, the presence of mechano-transduction elements that activates NADPH- oxidase 2 (Nox2)-dependent ROS generation during mechanical stress may also induce RyR1 defects (Ward et al. 2014). Moreover, this hypothesis is also supported by the beneficial effect of apocynin, a NADPH oxidase inhibitor, on diaphragm function in rats undergoing MV (McClung et al. 2009). Finally, in the rodent model of VIDD, 18 h of MV is considered as protracted (Powers et al. 2009), and very few data are available on the effects of longer MV periods on the diaphragm due to the rudimentary ventilation techniques used and the few possibilities to monitor vital functions and nutritional status. These limitations may introduce significant confounding factors when evaluating the effects of MV.

In a previous study using a similar piglet model, 5 days of MV induced loss of diaphragm fiber stiffness, suggesting an increased number of “non-functional” myosin or actin molecules due to myofibrillar disassembly and myofiber injury (Ochala et al. 2011). This could be caused by activation of calpains or caspases, two Ca²⁺-dependent proteolysis systems that are upregulated in rat diaphragm after mechanical ventilation (Shanely et al. 2002; Maes et al.

2007; McClung et al. 2007). These and our present results strongly suggest that the SR Ca^{2+} leak observed in the MV group could be involved in myofibrillar remodeling linked to Ca^{2+} -dependent proteolysis, ultimately leading to the muscle weakness observed in mechanically ventilated patients.

The finding by Ochala et al. (2011a, b) that in rats, calpain-1 activation is not increased before 9 days of MV could be in contradiction with our hypothesis on the early involvement of SR Ca^{2+} leakage and Ca^{2+} -stimulated myofibrillar remodeling in VID. However, this late calpain-1 activation was observed in hindlimb muscle, a different model of muscle unloading compared with the diaphragm. Indeed, in hindlimbs, muscle ‘disuse’ is mainly induced by contractile inactivity during MV. On the other hand, the diaphragm is concomitantly mechanically unloaded, electrically quiescent and undergoing changes in myofiber length caused by the cyclical lung inflation or positive end-expiratory pressure. Moreover, the diaphragm is the only muscle exposed to a negative-pressure environment along its pleural surface that can potentially serve as a stretch-like hypertrophic stimulus (Yang et al. 1998), which is removed by the application of positive-pressure ventilation. In addition, the diaphragm is more active than most other skeletal muscles (30–40% of the time, 24 h/day). All these factors could explain the very rapid diaphragmatic muscle atrophy and force loss observed during MV. Indeed, it has been estimated that 12 h of MV in rats is approximately equivalent to 96 h of locomotor muscle unloading in terms of induced muscle wasting responses (Powers et al. 2007). In line with these considerations, in humans, atrophy, injury and activation of Ca^{2+} -dependent proteolysis have been observed after a MV duration similar to the one used in the present study (Levine et al. 2008; Jaber et al. 2011).

The main objective of MV is to unload the respiratory muscle when the breathing work required to efficiently ventilate the lungs overcomes the performance of respiratory muscles. This unloading may be total (CMV) or partial (ASV). Previous studies have reported the beneficial effect of ventilation modes that only partially unload the diaphragm to prevent VID in small animal models (Gayan-Ramirez et al. 2005; Sassoon et al. 2004; Futier et al. 2008) and in a large animal model (Jung et al. 2010). However, the cellular mechanisms accounting for these beneficial effects are still poorly understood. We propose here, that during MV, the partial spontaneous activity of the diaphragm preserved in ASV might prevent RyR1 post-translational modifications induced by the total unloading of respiratory muscle. By only partially unloading the respiratory muscles, ASV might attenuate the energy homeostasis rupture and substrate oversupply to the mitochondria, described in VID (Picard et al. 2012). This beneficial

effect could prevent mitochondrial ROS production that then leads to RyR1 modification, Ca^{2+} homeostasis rupture and activation of Ca^{2+} -dependent proteolysis. This hypothesis needs now to be demonstrated experimentally.

Since VID description, clinicians have tried to develop alternative forms of MV, such as assist-control ventilation or pressure support ventilation, where a partial neuronal activation and mechanical activity of the diaphragm still exists. Although these new modes of ventilation support are now frequently used in the clinical practice (Esteban et al. 2000), few studies have evaluated their impact on the cellular pathways involved in VID. Our study brings new insights that might help understanding the beneficial effect on diaphragm function of these new MV modes that should be employed in ICU as often as possible to prevent VID. However, CMV is still commonly used in patients with acute respiratory distress syndrome in ICU, and cannot be efficiently replaced by ASV (Putensen et al. 2001). Thus, it is clearly in this particular context that clinical trials are needed to test small molecule drugs to stabilize RyR1 and counteract Ca^{2+} homeostasis defect.

We previously observed in humans after a similar period of CMV, the presence of diaphragm weakness associated with RyR1 defects (Jaber et al. 2011; Matecki et al. 2016). However, other mechanisms, including anesthetics, pain which could induce an overstimulation of the adrenergic response and consequently elevated levels of circulating endogenous catecholamines (Dunser and Hasibeder 2009; Boldt et al. 1995), have been associated with generalized myopathy in animal models (Ng et al. 2002) and could also explain these observations in patients on MV. The main advantage of the piglet model is the possibility to have a control group (ASV group) that only differs from the experimental model (MV group) by the level of diaphragm contractile activity. This was the only way to single out the unloading effect, in a similar context as observed in ICU. Thus, our study emphasizes the necessity of clinical trials to target specifically the unloading effect, the only parameters that cannot be removed, to prevent VID when CMV is necessary.

The main weakness of our study is the absence of any evidence on the causal link between diaphragm weakness and RyR1 defect. To make this link, the effect of small molecule drugs that stabilize RyR1 defect should be evaluated in this model, as previously done in the mouse model (Matecki et al. 2016).

In patients, diaphragm weakness induced by MV could have several other causes that might interfere with the unloading effect, such as metabolic disorders, sepsis, drug used for sedation, type of ventilation mode or comorbidity (Jung et al. 2010; Jaber et al. 2008; Sassoon et al. 2004; Divangahi et al. 2007; Testelmans et al. 2006). The relative contributions of each of these mechanisms of diaphragm

| Mouse model | Pig model | Patient |
|--------------------------------------|------------------|--------------|
| No Co-morbidity | | Co-morbidity |
| Short term MV | Long term (MV) | |
| No fiber remodeling | Fiber remodeling | |
| RyR1 dysfunction and muscle weakness | | |

Fig. 4 RyR1 dysfunction is observed in the different animal models of VIDD and also in patients, independently of the mechanical ventilation (MV) duration and the presence of confounding factors

weakness, which could cause failure to wean from the ventilator in ICU, are extremely difficult to evaluate in humans. Mechanically ventilated piglets represent a unique model that allows separating all these VIDD contributors, in a context very similar to clinical reality. Mechanically ventilated mice are an interesting tool to test easily new pharmacological treatments, or to better explore a particular cellular pathway involved in VIDD; however, larger animal models should be developed in parallel for pre-clinical studies of VIDD treatments. Indeed, RyR dysfunction and abnormal resting SR Ca²⁺ leak have been observed in different animal models and in mechanically ventilated human patients (Fig. 4). This abnormal pathway seems to be present since the early onset of VIDD with or without comorbidity and may participate to the mechanisms involved in muscular weakness.

A previous study demonstrated in a mouse model, that preventing RyR1 leak with S107, a small molecule drug known to stabilize RyR1-calstabin1 interaction, can prevent MV-induced muscle weakness and muscle fiber atrophy in mice (Matecki et al. 2016). Our study strongly supports the interest of a clinical trial in patients who require artificial respiratory support, using similar a compound to prevent the RyR1-mediated SR Ca²⁺ leak induced by MV.

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