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► **To cite this version:**

Haikel Dridi, Mohamad Yehya, Robert Barsotti, Steven Reiken, Claire Angebault, et al.. Mitochondrial oxidative stress induces leaky ryanodine receptor during mechanical ventilation. *Free Radical Biology and Medicine*, 2020, 10.1016/j.freeradbiomed.2019.11.019 . hal-02377213

**HAL Id: hal-02377213**

**<https://hal.science/hal-02377213>**

Submitted on 3 Jul 2020

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# Mitochondrial oxidative stress induces leaky ryanodine receptor during mechanical ventilation

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## ARTICLE INFO

### Keywords:

Calcium homeostasis

Mitochondrial oxidative stress

Diaphragm contractile function

## ABSTRACT

**Rationale:** Ventilator-induced diaphragm dysfunction (VIDD) increases morbidity and mortality in critical care patients. Although VIDD has been associated with mitochondrial oxidative stress and calcium homeostasis impairment, the underlying mechanisms are still unknown. We hypothesized that diaphragmatic mitochondrial oxidative stress causes remodeling of the ryanodine receptor (RyR1)/calcium release channel, contributing to sarcoplasmic reticulum (SR)  $Ca^{2+}$  leak, proteolysis and VIDD.

**Method:** In mice diaphragms mechanically ventilated for short (6 h) and long (12 h) period, we assessed mitochondrial ROS production, mitochondrial aconitase activity as a marker of mitochondrial oxidative stress, RyR1 remodeling and function,  $Ca^{2+}$  dependent proteolysis, TGF $\beta$ 1 and STAT3 pathway, muscle fibers cross-sectional area, and diaphragm specific force production, with or without the mitochondrial targeted anti-oxidant peptide d-Arg-2', 6'-dimethyltyrosine-Lys-Phe-NH<sub>2</sub> (SS31).

**Measurements and main results:** 6 h of mechanical ventilation (MV) resulted in increased mitochondrial ROS production, reduction of mitochondrial aconitase activity, increased oxidation, S-nitrosylation, S-glutathionylation and Ser-2844 phosphorylation of RyR1, depletion of stabilizing subunit calstabin1 from RyR1, increased SR  $Ca^{2+}$  leak. Preventing mROS production by SS31 treatment does not affect the TGF $\beta$ 1 and STAT3 activation, which suggests that mitochondrial oxidative stress is a downstream pathway to TGF $\beta$ 1 and STAT3, early involved in VIDD. This is further supported by the fact that SS-31 rescue all the other described cellular events and diaphragm contractile dysfunction induced by MV, while SS20, an analog of SS31 lacking antioxidant properties, failed to prevent these cellular events and the contractile dysfunction.

Similar results were found in ventilated for 12 h. Moreover, SS31 treatment prevented calpain1 activity and diaphragm atrophy observed after 12 h of MV.

This study emphasizes that mitochondrial oxidative stress during 6 h-MV contributes to SR  $Ca^{2+}$  leak via RyR1 remodeling, and diaphragm weakness, while longer periods of MV (12 h) were also associated with increased  $Ca^{2+}$ -dependent proteolysis and diaphragm atrophy.

## 1. Introduction

Mechanical ventilation (MV) is used clinically to achieve adequate pulmonary gas exchange in patients suffering from hypoxemia and in patients who are incapable of maintaining sufficient alveolar

ventilation (e.g. surgery, respiratory and heart failure, drug overdose). However, prolonged MV results in significant diaphragmatic weakness due to both myofiber atrophy and contractile dysfunction [1]. This condition, which has been termed ventilator-induced diaphragmatic dysfunction (VIDD) [2], plays an important role in the difficulties

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frequently encountered in weaning critically ill patients from the ventilator and increased comorbidities [3].

Although the precise cellular pathways involved in VIDD remain poorly understood, mitochondrial oxidative stress seems to play a major role [4–8]. This was confirmed by a recent study which showed that SS31 (D-Arg-2', 6'-Dimethyl-Tyr- Lys-Phe-NH<sub>2</sub>) a mitochondrial antioxidant protects the diaphragm from MV-induced diaphragmatic atrophy and contractile dysfunction in rats [6]. Moreover, Picard et al. reported the presence of intramyofibrillar (IMF) mitochondrial fission after only 6 h of MV [9] in mice, which may be linked to an increase of mitochondrial ROS production.

In contrast, a recent study revealed that diaphragm muscle fibers from mechanically ventilated patients display significant atrophy and contractile dysfunction in the absence of major mitochondrial alterations and oxidative stress [10]. This discrepancy needs to be further investigated in order to better establish the role of mitochondria in the pathogenesis of VIDD and identify an effective therapeutic target. To date, the downstream cellular pathways activated by mitochondrial oxidative stress, as well as its link with early events previously involved in the genesis of VIDD, such as calcium homeostasis impairment or activation of the JAK (Janus kinase) and STAT (signal transducer and activator of transactivation) signaling pathway are unknown. Sarcoplasmic reticulum (SR) Ca<sup>2+</sup> release channel/ryanodine receptors (RyR1), responsible for the release of Ca<sup>2+</sup> required for excitation–contraction (EC) coupling, contributes to the pathophysiological signaling in VIDD. Indeed, in previous studies in mice, we observed that only 6 h of MV were sufficient to cause RyR1 alterations due to a synergistic effect of RyR1 oxidation and PKA phosphorylation, leading to diaphragm force deficit, without atrophy [11,12].

The JAK-STAT pathway is stimulated early during mechanical ventilation, that in-turn, activates MV-induced oxidative stress and represents a key mechanism in the development of VIDD [13,14]. Alternatively, oxidative stress may directly activate the JAK-STAT pathway [15] and thus could initiate a vicious cycle to promote VIDD. Whether or not these 2 early factors involved in VIDD are linked through a common activator, or can be considered as independent factors is unknown. The goal of this study was to determine if mitochondrial oxidative stress acts as a trigger or as a consequence of these early mechanisms previously described.

The present study was designed to determine for the first time, whether either mitochondrial stress and/or activation of JAK-STAT pathway contributes to the early remodeling of RyR1 and Ca<sup>2+</sup> homeostasis impairment, leading to contractile dysfunction and whether a longer period of MV this mechanism eventually causes calcium-dependent proteolysis and diaphragm atrophy that has been observed in animal models after 12 h of MV or 48 h in humans [1,5,10,16–18].

Thus we evaluated, the relationships among mitochondrial oxidative stress, RyR1 remodeling, JAK-STAT pathway activation, diaphragm dysfunction and atrophy in mice treated with a mitochondria-targeted antioxidant SS31 and ventilated during 6 or 12 h in comparison with non-treated groups mechanically ventilated.

## 2. Methods

### 2.1. Experiments on animals and institutional approval

The study followed the guidelines for animal experiments established by the institutional committee for animal care (Montpellier, France) and the recommendations of the World Medical Association. It was recorded under the reference #CEEA-LR-12078. Healthy male (10–12 weeks, 25–30 g) C57/BL6 mice were maintained in our animal facility for 1 week before initiation of the experiments.

### 2.2. Experimental design

Mice were then randomly assigned to one of five experimental

groups: 1/mice mechanically ventilated for 6 h (MV6h, n = 8), 2/mice mechanically ventilated for 6 h and treated by SS31 (loading dose 3 mg/kg and maintaining dose 0.05 mg/kg/h during MV) (MV6h + SS31, n = 8), 3/mice mechanically ventilated for 12 h (MV12h, n = 6), 4/mice mechanically ventilated for 12 h and treated with SS31 (loading dose 3 mg/kg and maintaining dose 0.05 mg/kg/h) (MV12h + SS31, n = 6) and 5/control mice (Control, n = 8). SS31 molecule was dissolved in Ringer's lactate solution (RL). RL was used as vehicle for group 1, 3 and 5. To be sure that the effects of SS31 are due to its antioxidant properties a group of 6 mice was ventilated for 6 h and treated with SS20, an analog of SS31 which does not target the mitochondria and does not exert any antioxidant effect.

### 2.3. Mitochondria-targeted antioxidant: chemical details and experimental delivery

The peptides, SS-31 and SS-20, used in these studies were supplied in powder form (purity > 99%) by Genemed Synthesis, Inc. (San Antonio, TX). SS31 and SS20 were dissolved in RL and delivered in a bolus (loading) dose (3 mg/kg, intraperitoneal (IP) injection) 15 min before initiation of MV. A constant IP injection (0.05 mg/kg/h) of SS31 or SS20 was maintained throughout MV.

### 2.4. Mechanical ventilation

Mice in the MV6h or MV12h groups were anesthetized, intubated and mechanically ventilated as previously described [11]. More details are described in the Supplementary information (SI).

### 2.5. Mitochondrial ROS production and aconitase activity assay

Mitochondria were isolated from diaphragm and mitochondrial ROS production and aconitase activity was measured as previously described [19] and detailed in the SI.

Briefly, mitochondrial fractions (50 µg) were incubated in VO<sub>2</sub> buffer (250 mM sucrose, 50 mM KCl, 25 mM Tris-HCl, and 10 mM K<sub>2</sub>HPO<sub>4</sub>, pH 7.4) in a 96-well black plate. ROS production was assessed at 37 °C for 60 min during states 3 and 4 respiration, by adding respiration substrates before the addition of 50 µM dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA, Invitrogen). ROS production is directly proportional to fluorescence emission monitored at ex 485 nm/em 528 nm with a microplate fluorimeter. Microplate data were compiled and analyzed using i-control™ Microplate Reader Software (version 1.11), and results were expressed as arbitrary fluorescent units.

### 2.6. RyR1 immunoprecipitation

To determine the oxidation, phosphorylation, nitrosylation, glutathionylation and calstabin1 binding to RyR1, immunoprecipitation was performed in diaphragm biopsies with an RyR1 specific antibody (2 µg) in 0.5 ml of a modified radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.2, 0.9% NaCl, 5.0 mM NaF, 1.0 mM Na<sub>3</sub>VO<sub>4</sub>, 1% Triton X-100, and protease inhibitors) overnight at 4 °C and incubated with protein A-Sepharose beads (Sigma) at 4 °C for 1 h as previously described [12,20] and detailed in the SI.

### 2.7. PKA activity assay

PKA activity was determined by using a colorimetric assay (Arbor assay: K027H1) according to manufacturer's instructions and previously described (See SI) [12].

### 2.8. Western blotting and enzymatic calpain activity

Immunoblotting was performed in diaphragm by standard methods using antibodies against: calpain1 (15C10, 1: 1000 dilution, Sigma),

GAPDH (Ab8245, 1: 60,000 dilution, Abcam), TGF $\beta$ 1 (ab64715, 1/ 1,1000 dilution, Abcam), STAT3 (ab119352, 1:1,1000 dilution, Abcam), STAT3 phospho Y705 (ab76315, 1:1, 000 dilution, Abcam). The enzymatic activity of calpain-1 was measured using Calpain Activity Assay Kit (Abcam) according to manufacturer's instructions. More details are described in the SI.

## 2.9. Measurement of diaphragm contractile properties

The mice were euthanized by cervical dislocation. The costal diaphragm was surgically removed to permit measurements of isometric contractile properties as previously described [11,12]. More details are described in the SI.

## 2.10. Measurement of atrophy in diaphragm after 12 h MV

Cross sectional areas and fibers types distribution on diaphragm were measured using immunostaining technique with anti-myosin heavy chain, slow and fast. Muscle membrane was counterstained with dystrophin antibodies as previously reported [21].

## 2.11. Ca<sup>2+</sup> sparks measurement

Bundles of 4–7 fibers were manually dissected from diaphragm muscle in a relaxing solution (in mM: K-glutamate 140, Hepes 10, MgCl<sub>2</sub> 10, EGTA 0.1, pH 7.0), mounted as previously described [22] and permeabilized in the relaxing solution containing 0.01% saponin for 30–40 s. RyR1 leak was assessed by measuring spontaneous SR Ca<sup>2+</sup> release events (i.e, Ca<sup>2+</sup> sparks) in permeabilized muscle diaphragm fibers using laser scanning confocal microscope [16,18]. More details are described in the SI.

## 2.12. Statistical analysis

Data are expressed as mean  $\pm$  SEM. Statistical significance was defined as  $p < 0.05$ , using Student's unpaired *t*-test or analysis of variance (one-way or two-way), followed by a Bonferroni selected comparison test.

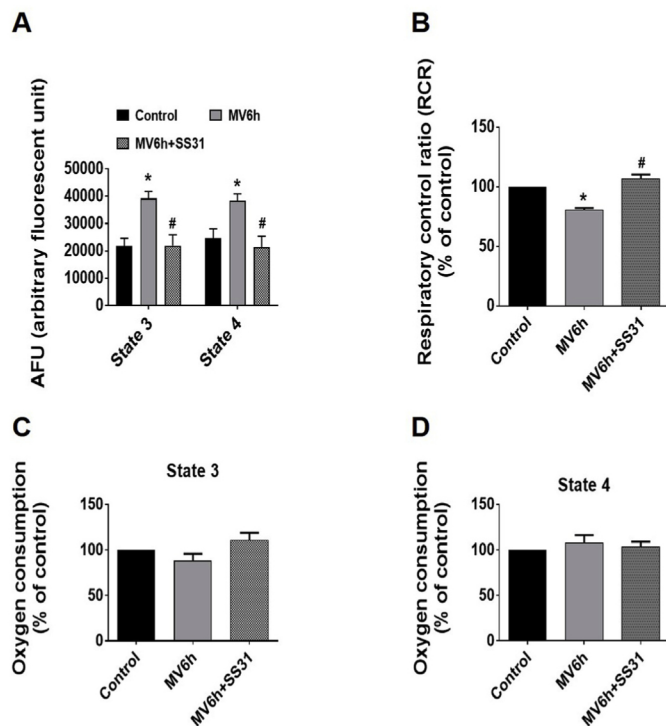
## 3. Results

### 3.1. of MV increases mitochondrial oxidative stress, RyR1 remodeling and diaphragmatic contractile dysfunction

To determine the effect of the mitochondrial antioxidant SS31 on diaphragmatic mitochondrial ROS production and respiration, we measured the mitochondrial ROS emission and the mitochondrial respiratory rate in control, MV6h and MV6h + SS31. Our data indicate that treatment with SS31 prevented MV-induced-increase in diaphragmatic mitochondrial ROS release during both state 3 and 4 respiration (Fig. 1A).

Mitochondrial ROS release induced by 6 h of MV is associated to a statistical reduction of the mitochondrial respiratory control ratio (RCR) without statistical effect in the rates of state 4 or state 3 respiration (Fig. 1B,C,D). It has been established that MV results in damage to mitochondria as indicated by impaired coupling (i. e, lower respiratory control ratio (RCR)) in mitochondria isolated from MV diaphragm [23]. Herein, SS31 was successful in averting diaphragmatic mitochondrial uncoupling that occurs following 6 h of MV (Fig. 1B).

To confirm our previous observation, mitochondrial oxidative stress was assessed by mitochondrial aconitase activity (measurement on isolated diaphragmatic mitochondria), RyR1 remodeling and dysfunction (as a downstream target of mitochondria ROS production) in murine diaphragms of control ( $n = 6$ ), MV6h ( $n = 6$ ) and MV6h + SS31 ( $n = 6$ ) groups. These animal were randomly chosen among the 8 animal previously described in the experimental design.



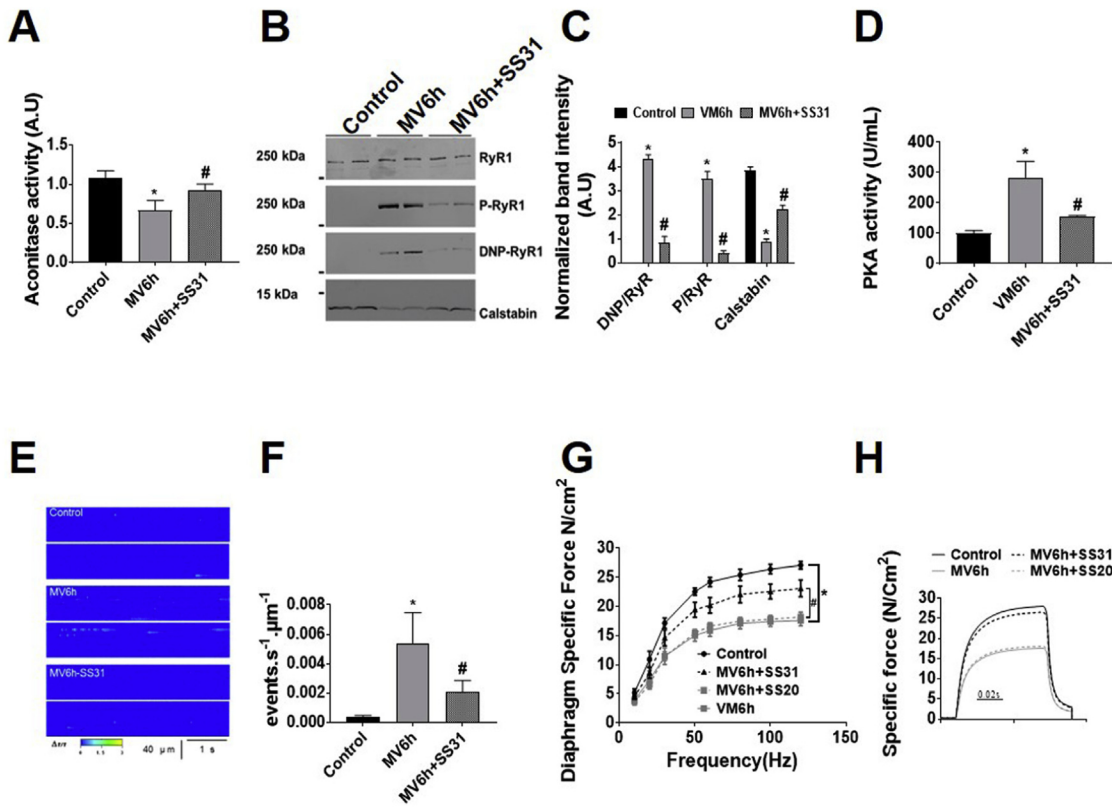
**Fig. 1.** Mitochondrial targeted antioxidant SS31 prevented mitochondrial ROS production and respiratory uncoupling. **A)** Rate of ROS emission from mitochondria isolated from controls, MV6h and MV6h + SS31 diaphragms. Results are expressed as mean  $\pm$  SEM,  $n = 6$  in control, MV6h and MV6h + SS31 groups (\* $P < 0.05$  MV6h vs. control, #, MV6h vs MV6h + SS31). **B)** Respiratory control ratio (RCR) in mitochondria isolated from diaphragms of control, MV6h and MV6h + SS31. **C, D)** State-3 respiration and State-4 respiration in mitochondria isolated from diaphragms of control, MV6h and MV6h + SS31. These data were obtained using pyruvate/malate as substrate. Results are normalized to the control.  $n = 6$  in control, MV6h and MV6h + SS31 groups (\* $P < 0.05$  MV6h vs. control, #, MV6h vs MV6h + SS31).

We observed that mitochondrial aconitase activity was decreased in MV6h group, which is in accordance with an increased mitochondrial oxidative stress (Fig. 2A). MV caused a significant increase in RyR1 S-nitrosylation, S-glutathionylation (Fig. S1), oxidation and phosphorylation on RyR1-Ser-2844 and calstabin1 dissociation from RyR1 (Fig. 2B and C). Protein Kinase A (PKA) activity was increased at 6 h of MV (Fig. 2D). The Ca<sup>2+</sup> sparks frequency is commonly used as an index of RyR1-mediated SR Ca<sup>2+</sup> leak consistent with the biochemical remodeling of the channel [16,18]. These biochemical abnormalities were associated with an increased frequency of SR Ca<sup>2+</sup> release events, evaluated *in situ* by measuring spontaneous RyR1 Ca<sup>2+</sup> sparks. (Fig. 2E and F). Each of these effects were prevented by the mitochondria-targeted antioxidant SS31.

As previously described by our laboratory [11], 6 h of MV in mouse resulted in a significant decrease of diaphragmatic force production at all stimulation frequencies (Fig. 2G), with a loss of 40% of force production at 120 Hz (Fig. 2H) ( $P < 0.001$ ) and without measurable changes in the amount of proteolysis or diaphragm cross sectional area after 6 h of MV [11]. This decrease in force was prevented by SS31 (Fig. 2G and H).

To ascertain the mitochondrial antioxidant properties of SS31, we used its inactive analogue SS20 (Phe-D-Arg-Phe-Lys-NH<sub>2</sub>) as a control and investigated its effect on VIDD after 6 h. As shown in (Fig. 2G and H) SS20 had no effect on diaphragmatic force generation in mice undergoing MV. The antioxidant properties of SS31 is mediated via the dimethyl tyrosine residue in its sequence. Since the dimethyl tyrosine residue in SS31 is not present in SS20 (Phe-D-Arg-Phe-Lys-NH<sub>2</sub>) this peptide does not display antioxidant properties thus supporting for the





**Fig. 2.** Reducing mitochondrial oxidative stress with the SS31 (selective mitochondrial antioxidant) prevents VIDD after 6 h of MV. **A**) Mitochondrial aconitase activity from mouse diaphragm of the three experimental groups: control, MV6h, and MV6h + SS31. **B**) Representative immunoblots of immunoprecipitated RyR1 from mouse diaphragm samples of the three experimental groups (control, MV6h, MV6h + SS31) (Each blot corresponds to adjacent wells of the same gel). Bar graphs **C**) show quantification of immunoblots, relative to total RyR1 immunoprecipitated. Results are expressed as mean  $\pm$  SEM, n = 6 in control, MV6h and MV6h + SS31 groups (\*P < 0.05 MV6h vs. control, #, MV6h vs MV6h + SS31). **D**) PKA activity was measured in diaphragm muscles in mice of the three experimental groups (control, MV6h, MV6h + SS31). Data are expressed as PKA activity in units per mL, quantified by using a colorimetric assay. Results are expressed as mean  $\pm$  SEM, n = 6 in control, MV6h and MV6h + SS31 groups (\*P < 0.05 MV6h vs. control, #, MV6h vs MV6h + SS31). **E**) Representative  $\Delta F/F$  line-scan images (1.5 ms per line) recorded in control, after MV6h and after MV6h with SS31 treatment. **F**) Comparison of mean  $Ca^{2+}$  sparks frequency between control, MV6h and MV6h + SS31 groups. Results are expressed as mean  $\pm$  SEM, n = 6 in control, MV6h and MV6h + SS31 groups (\*P < 0.05 MV6h vs. control, #, MV6h vs MV6h + SS31). **G**) Diaphragm muscle-specific force–frequency relationships measured *ex-vivo* in control, MV6h, MV6h + SS31, and MV6h + SS20 groups (n = 8 in each group, n = 6 in SS20 group). SS20 is an analog of SS31, which does not target the mitochondria and does not exert any antioxidant effects. (\*P < 0.05 MV6h vs. control, #, MV6h vs MV6h + SS31). **H**) Representative records of diaphragmatic specific force production measured *ex-vivo* at 120 Hz in muscle bundles under isometric conditions in control, MV6h, MV6h + SS31 and MV6h + SS20 groups.

first time, the hypothesis that increased mitochondrial oxidative stress induced by 6 h MV contributes to RyR1 structural and functional remodeling and contractile dysfunction of the diaphragm.

### 3.2. Mitochondrial oxidative stress stimulated by 12 h of MV in mice induces, $Ca^{2+}$ -dependent proteolysis and diaphragm weakness

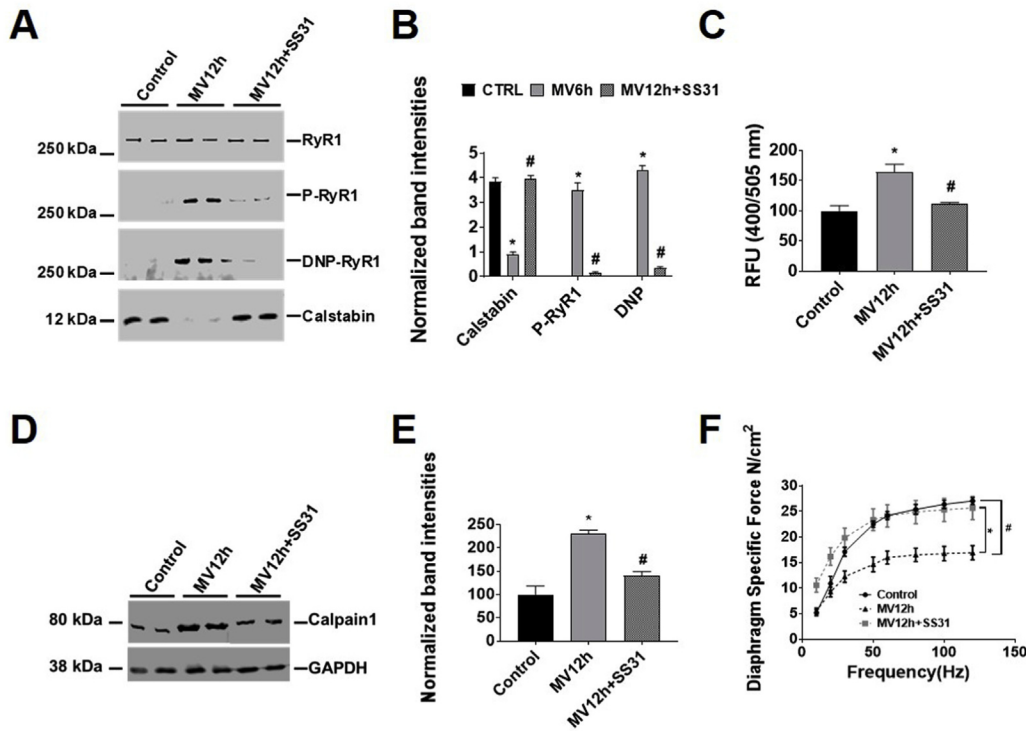
The mice were ventilated for 12 h (Fig. 3). Similarly, as described after 6 h of MV, we observed after 12 h of MV, decreased RyR1 S-nitrosylation, S-glutathionylation (see SI.1C, D) phosphorylation, oxidation and an increased calstabin1 binding to RyR1 with the SS31 treatment (Fig. 3A and B). To address the link between the early  $Ca^{2+}$  leak and the subsequent  $Ca^{2+}$  dependent proteolysis we measured the  $Ca^{2+}$  dependent protease (Calpain1) activity and levels by fluorometric assay and Western blotting. We found that only 12 h of MV resulted in a significant increase in both expression and activity of calpain1 in the ventilated diaphragm by 100%, p < 0.05 (Fig. 3C, D, E), associated with diaphragm weakness (Fig. 3F). As shown by Fig. 3, the RyR1 remodeling, calpain1 activation and diaphragm weakness due to 12 h of MV was abolished by SS31 treatment.

### 3.3. Mitochondrial oxidative stress stimulated by 12 h of MV in mice induces diaphragm atrophy

To determine the effect of 12 h of MV on diaphragmatic fibers size, we stained the diaphragm muscle fibers with antibodies against slow and fast myosin heavy chains. As shown in Fig. 4A, slow fibers (left panel) were distributed sporadically among the fast muscle fibers (right panel). 12 h of MV did not change the fiber distribution in the diaphragm. It was approximately 10% slow fibers and 90% fast muscle fibers in this strain of mice (C57BL/6), (Fig. 4A, E). However, the mean CSA was significantly reduced (Fig. 4B and C) due mainly to a reduction of CSA fast fibers (Fig. 4D). Although CSA in slow muscle fibers was also reduced by 10%, the difference was not statistically significant (p = 0.13).

### 3.4. The JAK-STAT pathway is not activated by MV induced mitochondrial oxidative stress

Thus, to identify whether or not mitochondrial oxidative stress activate the JAK-STAT pathway, we evaluated the effect of SS-31 treatment on the expression of TGF $\beta$ 1, STAT3 and its relative phosphorylation in our mechanically ventilated mice. We observed that 6 and 12 h of MV significantly increased TGF $\beta$ 1 expression and STAT3



**Fig. 3.** Reducing mitochondrial oxidative stress with the SS31 (selective mitochondrial antioxidant) prevents RyR1 remodeling induced VIDD after 12 h of MV. **A)** Representative immunoblots of immunoprecipitated RyR1 from mouse diaphragm of the three experimental groups: control, MV12h, and MV12h + SS31. (Each blot corresponds to adjacent wells of the same gel). **B)** Bar graphs show quantification of immunoblots, relative to total RyR1 immunoprecipitated. Results are expressed as mean  $\pm$  SEM,  $n = 6$  in each group (\* $P < 0.05$  MV12h vs. control, #, MV12h vs MV12h + SS31). **C)** The mean  $\pm$  SEM of calpain activity levels in relative fluorescent units in control, MV12h and MV12h + SS31 groups. Results are presented as a percent of controls. (\* $P < 0.05$  MV12h vs. control, #, MV12h vs MV12h + SS31). **D)** Representative immunoblots of calpain 1 expression from mouse diaphragm of the 3 experimental groups: control, MV12h, MV12h + SS31 (Each blot corresponds to adjacent wells of the same gel). **E)** Bar graphs show quantification of immunoblots, relative to total GAPDH. **F)** Diaphragm muscle-specific

force–frequency relationships measured *ex-vivo* in control, MV12h and MV12h + SS31 groups ( $n = 6$  in each group) (\* $P < 0.05$  MV12h vs. control, #, MV12h vs MV12h + SS31).

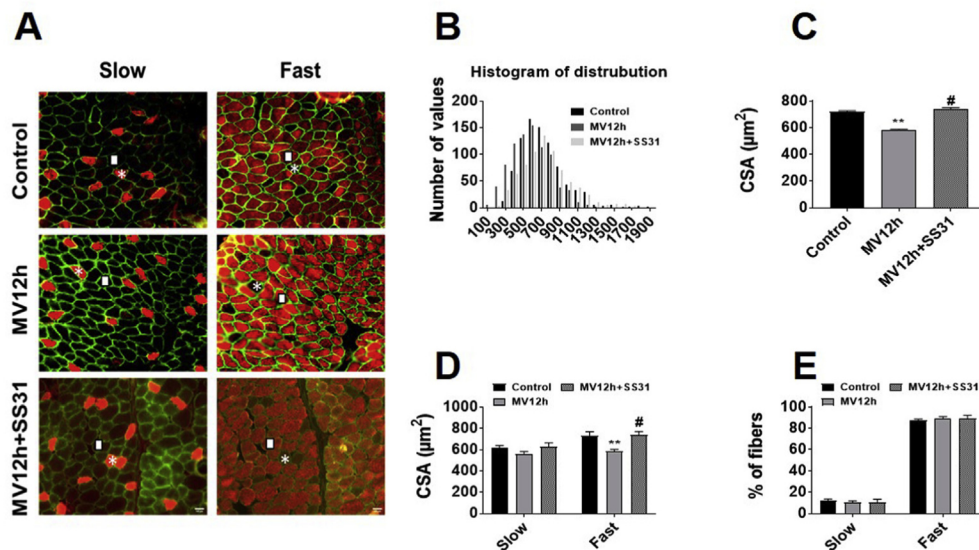
phosphorylation, however, we didn't observe any preventive effect of SS31 treatment on the activation of this early pathway involved in VIDD (Fig. 5A, B, C and D).

#### 4. Discussion

Several upstream signals such as Calcium homeostasis impairment, mitochondrial oxidative stress or activation of the JAK-STAT have been previously identified as early events leading to VIDD. However, the link unifying them is unclear, which may falsely lead to consider them as independent factors reducing the efficacy of any therapeutic

intervention.

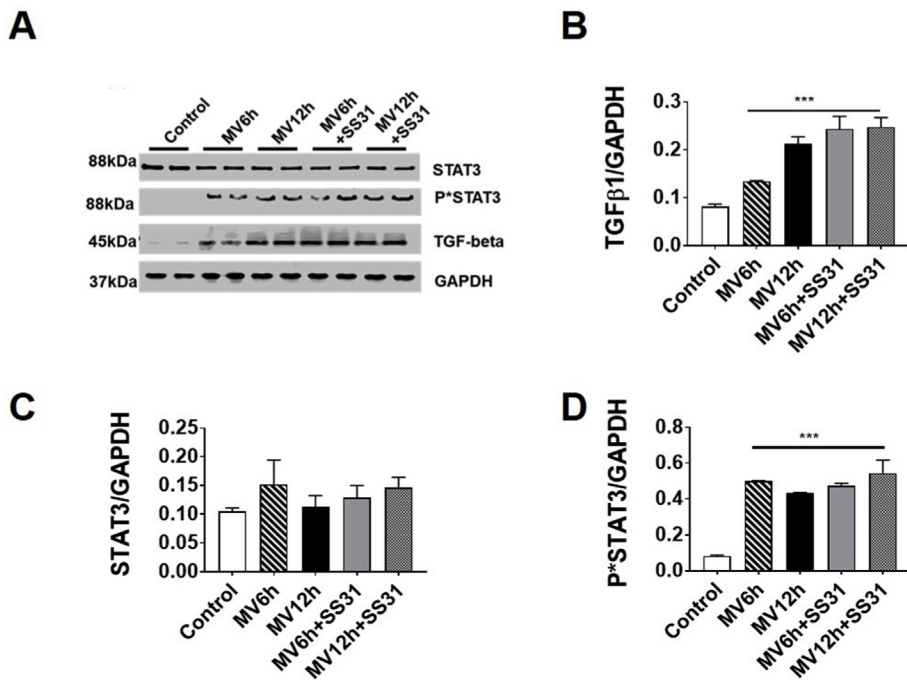
In the present study we report, for the first time, in diaphragm mice mechanically ventilated during 6 h, that MV induced mitochondrial oxidative stress early contributes to the biochemical signature (remodeling) of leaky RyR1 channels, leading to intracellular  $Ca^{2+}$  leak and diaphragm weakness. Mitochondrial oxidative stress appears as an upstream signal behind the calcium homeostasis impairment. In our study, mitochondria were isolated and used to evaluate the mitochondrial ROS production, respiration and mitochondrial aconitase activity as a biomarker of mitochondrial oxidative stress. Aconitase activity in mitochondria has been reported to be a sensitive redox sensor of



**Fig. 4.** Reducing mitochondrial oxidative stress with the selective mitochondrial antioxidant SS31 prevents atrophy after 12 h of MV. **A)** Representative image of cryosections of mouse diaphragm showing fast and slow muscle fibers by immunostaining with antibodies against fast (right panel, red) and slow (left panel, red) myosin. Fibers membrane are staining in green using dystrophin. **B)** Distribution histogram of muscle fiber cross-sectional area (CSA) from a sample of  $\sim 900$  fibers per control, MV12h and MV12h + SS31 diaphragm sample ( $n = 6$  in each group). Mean CSA was measured by the image processing software Fiji. **C)** Mean CSA in the hole diaphragm from each of the 3 groups (control, MV12h and MV12h + SS31). **D)** Cross section area in separated slow and fast diaphragmatic fiber in the 3 groups. **E)** Percentage of slow and fast fibers in mouse diaphragm from the 3 groups. Muscle membrane was counter-stained with dystrophin antibodies. White

squares indicate fast fibers, whereas the asterisks show the slow fibers in consecutive sections. (\* $P < 0.05$  MV12h vs. control, #, MV12h vs MV12h + SS31). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)





**Fig. 5.** Reducing mitochondrial oxidative stress with the selective mitochondrial antioxidant SS31 doesn't prevent TGFβ1, STAT3 and Phospho-Stat3 activation in mechanically ventilated mice during 6 or 12 h. (A) Representative immunoblots of TGFβ1, STAT3 and Phospho-Stat3 from mouse diaphragm samples of the 5 experimental groups: control, MV6h, MV12h, MV6h + SS31, MV12h + SS31. (Each blot corresponds to adjacent wells of the same gel.) Bar graphs (B) show quantification of immunoblots, relative to GAPDH. Results are expressed as mean ± SEM, (n = 6 in each group), (\*P < 0.05 vs. control).

reactive oxygen species and nitrogen species in cells [24,25]. In fact, loss in aconitase activity is commonly used as a biomarker of oxidative damage due to the susceptibility of the enzyme's [4Fe-4S]<sup>2+</sup> cubane cluster to oxidative disassembly [26,27]. We also report with longer period of MV (12 h), the appearance, of Ca<sup>2+</sup> dependent proteolysis and atrophy, which are linked to similar cellular events observed after short period of MV (6 h).

We previously reported that RyR1 remodeling is involved in VIDD pathogenesis [12] occurring prior to proteolysis or atrophy [11]. However, the link between mitochondrial oxidative stress and RyR1 leak during VIDD has not previously been established. As described [12], we observed that 6 h of MV induces S-nitrosylation, S-glutathionylation, oxidation and phosphorylation at Ser-2844 of diaphragm RyR1, associated with a loss in the interaction between RyR1 and the stabilizing subunit calstabin1 involved in the functional interaction between RyR1 and the voltage-sensing Ca<sup>2+</sup> channel (DHPR) [28]. The result is a destabilization of the closed state of the Ca<sup>2+</sup> channel resulting in SR Ca<sup>2+</sup> leak, and impaired excitation-contraction coupling (ECC) with reduced maximal SR Ca<sup>2+</sup> release [29]. RyR1 oxidation has been causally implicated in the reduction of specific force generating capacity in mammalian skeletal muscle [16]. In aging, mitochondrial oxidative stress was identified as an important mediator of RyR1 oxidation and the related muscle dysfunction [30]. The functional consequence of RyR1 oxidation is SR Ca<sup>2+</sup> leak and depleted Ca<sup>2+</sup> stores that lowers tetanic Ca<sup>2+</sup> and weakens muscle force production [31]. Beside oxidation, RyR1 can undergo others redox modification such as S-nitrosylation/S-glutathionylation [32–34]. RyR1 contains multiple cysteine residues that can be modified at physiological pH by either S-nitrosylation [32,35] or S-glutathionylation [34]. cGMP-independent, NO-mediated regulation of RyRs increases channel activity in vesicles and in single channel measurement [35]. Exogenous S-nitrosylation of RyR1 has been shown to reduce the affinity of calstabin1 binding to RyR1 [36]. We have found increased S-nitrosylation in our MV mice compared to controls consistent with calstabin1 dissociation and increased calcium sparks through RyR1 channel and the subsequent diaphragmatic weakness. These findings are in accordance with previous studies showing S-nitrosylation causing muscle weakness in muscle dystrophy [18]. However, we have found slight increase in RyR1 S-glutathionylation in our MV mice. Therefore, it seems like the S-

glutathionylation is not a major modification of RyR1 in the onset of the VIDD.

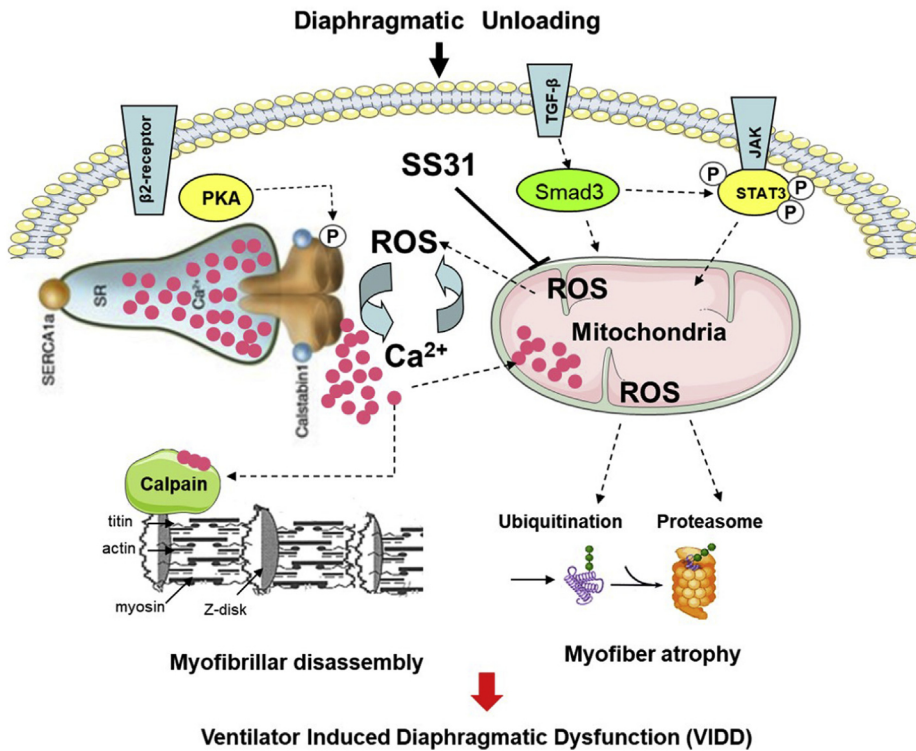
We also observed in MV mice an increased PKA activity which could explain the RyR1 PKA hyper-phosphorylation as demonstrated in our previous study [12]. Indeed, oxidative stress has been shown to increase the activity of several protein kinases, including PKA, PKC, PKG and CaMKII, and to reduce the activity of protein phosphatases [37]. Moreover, PKA is redox sensitive, forming an interprotein disulfide bond between its two regulatory subunits in response to cellular hydrogen peroxide. This oxidative disulfide formation causes a subcellular translocation and activation of the kinase resulting in phosphorylation of established substrate proteins [38].

To determine whether MV-induced mitochondrial oxidative stress caused an increase in RyR1-mediated SR Ca<sup>2+</sup> leak, we evaluated the preventive effect of SS31, a mitochondria-targeted antioxidant, on RyR1 remodeling and spontaneous Ca<sup>2+</sup> release events (i.e. Ca<sup>2+</sup> sparks) in murine diaphragm ventilated for 6 h.

The antioxidant SS31, that selectively binds to cardiolipin via electrostatic and hydrophobic interactions to protect the structure of mitochondrial cristae and oxidative phosphorylation [39], prevented the increase in PKA activity, RyR1 PKA phosphorylation and oxidation and the subsequent loss of diaphragmatic force production and atrophy in ventilated mice. Moreover, as showed (Fig. 2G and H) we observed that SS-20 treatment (inactive analog of SS31) [40], which has the same structure as SS31 but without the antioxidant radical, does not prevent VIDD. This result highlights the specific role of mitochondrial oxidative stress in the pathogenesis of VIDD.

Our result suggests that MV causes a vicious cycle of RyR1 leak and mitochondrial oxidative stress during VIDD. The initiating signal during MV is likely due to the TGFβ and/or Jack-stat pathway [13]. It's a signaling cascade that can be activated by cytokines, hormones and growth factor via ligand-receptor interaction. In our mechanically ventilated mouse model during 6 or 12 h, expression of TGFβ1, STAT3 and phosphoSTA3 is increased without any preventive effect of SS31 treatment (Fig. 5). These data suggest that TGFβ and JAK-STAT pathway activation are not targeted by mitochondrial oxidative stress and could be considered as an upstream signaling pathway of mitochondrial oxidative stress and RyR1 remodeling.

Mechanical stress could be a potential activator of TGFβ and JAK-



**Fig. 6.** Several of the mechanistic pathways implicated in the development of VIDD are illustrated. TGF $\beta$  activates Smad3 and induces mitochondrial ROS production due to diaphragm unloading during MV. Jack-Stat pathway activation increases mitochondrial ROS production. ROS generation from mitochondria can oxidize ryanodine receptor leading to calstabin1 depletion induced calcium leak and can activate downstream proteolytic pathways involved in myofiber injury and atrophy (calpains). SS31 scavenges radical species in the inner part of mitochondria and protect posttranslational modifications of RyR1 inducing calcium leak and calpains activation.

STAT pathway. Indeed, the skeletal muscle including the diaphragm is a mechanically sensitive structure due to the mechanotransducing proteins and complexes located on its membrane like the G-coupled protein receptor, the dystrophin-associated glycoprotein complex and the integrin [41,42]. These structures span the cellular membrane physically connecting the extracellular matrix and the cytoskeleton, allowing mechanical signal transmission, which may be involved, in several pathophysiological process including gene transcription, calcium and ROS regulation [43,44]. However, their implication in VIDD in regard to diaphragm unloading associated to mechanical stretch due to asynchrony between the patient and its ventilator is poorly known. Recently, Zhang et al. provided an important link between mechanical load-sensing via the dystrophin-associated protein complex and the protein synthesis/degradation pathways in unloaded muscle which implicated the TGF $\beta$  network [45]. In addition, TGF $\beta$ 1 has been demonstrated to be electrical activity-dependent in cultured myotubes [46]. However, the link between diaphragmatic unload, TGF $\beta$ 1 activation and mitochondrial ROS production oxidative stress induced VIDD remains to be elucidated in further studies.

After longer periods of MV (12 h), our study suggests that dysregulated Ca<sup>2+</sup> homeostasis induced by mitochondrial oxidative stress which already initiates after 6 h of MV may trigger Ca<sup>2+</sup>-dependent proteolysis and thus atrophy as previously described [5,47,48]. Indeed, the finding that SS31 prevents activation of calpain1 after 12 h of MV is consistent with numerous previous reports suggesting a regulatory role of oxidative stress in the stimulation of proteolytic system, which contribute to atrophy [1,5,49–52]. The activation mechanisms involved remain to be demonstrated. Indeed, although mitochondrial oxidative stress may directly activate these proteolytic systems [53–55], dysregulation of intracellular Ca<sup>2+</sup> homeostasis observed in our MV12h group is also a possible key activator [56–58]. Whether this mitochondrial oxidative stress-induced Ca<sup>2+</sup> leak triggers proteolysis as a predominant mechanism is an important question for future research.

MV-induced atrophy has been reported following more than 24 h in humans [1,59,60], and 12 h in rats or mice [10,17,61]. Consistent with these studies, we observed a reduction of cross-sectional area (CSA) of diaphragm myofibers in the MV12h group without any change in the

distribution of fiber types. Interestingly, we found that after 12 h of MV in mice, only fast fibers exhibited a significant degree of atrophy, while the decrease in CSA of the slow type was not statistically significant. This result suggests a higher susceptibility of diaphragm fast fibers to atrophy induced by MV. The reason is unclear but it could be related to a higher susceptibility of fast fiber types to atrophy when subjected to eccentric contractions due to ventilator/mouse asynchrony [62–64]. Although this is an interesting theory, further studies are needed to ascertain whether mechanical stress induced by MV preferentially damages fast fibers.

Finally, Den Berg et al. [10] have reported diaphragm weakness and atrophy with preserved mitochondrial function and morphology in ventilated critically ill patients which is controversial to our and other previous finding [9,23,59,65]. In fact, our animal model was slightly ventilated under hypocapnia to inhibit central respiratory drive and diaphragm contractile activity. This is not the case in critically ill patients, where a certain level of contractile activity is preserved, that could explain the preserved mitochondrial function observed [10]. Indeed, in patients undergoing cardiothoracic surgery, it was demonstrated that even a very low level of diaphragm contractile activity induced by magnetic stimulus, at a much lower frequency than normal breathing is sufficient to protect against mitochondrial dysfunction and oxidative stress [66,67].

Moreover, we previously observed in mice, that mechanical ventilation with chronic positive airway pressure maintains some diaphragm contractility and prevents diaphragm from RyR1 remodeling induced by oxidative stress [12]. This discrepancy between critically ill patients and animal models, highlights the importance of maintaining diaphragmatic contractile activity during mechanical ventilation to prevent VIDD.

#### 4.1. Limits of our findings

In the present study, we evaluated ROS release from mitochondria and, therefore estimating the total mitochondrial superoxide production, which depends on diaphragmatic mitochondrial ROS scavenging capacity. Previous study observed in similar animal model, that MV



increases the mitochondrial protein level of both GPX1 and catalase without any effect on SOD [23]. This result suggests that in our model, the increase of mitochondrial ROS release is not the result of a decrease of mitochondrial scavenging capacity but an increase of mitochondrial ROS production.

We also did not address the direct link between the physical changes associated with diaphragm disuse during MV and the subsequent molecular alterations. Previous studies have reported that Smad3 as an activity-dependent sensor which responds rapidly to MV-induced muscle unloading by altering its phosphorylation. Thus Smad3 activity regulates proteins degradation and mitochondrial dysfunction/oxidative stress. Smad3 appears to be an upstream signal to activated the Jack-STAT pathway since Smad3 activation occurs earlier after the institution of MV than does STAT3 phosphorylation and Smad3 inhibition leads to inhibition of STAT3 [13,68]. It is possible that Smad3 and Jack/STAT signaling may govern common downstream targets for example NOX4-mediated mitochondrial dysfunction/oxidative stress [69,70]. However, Ca<sup>2+</sup> may be a regulator of Smad3 since muscle activity regulates Ca<sup>2+</sup> release. In the present study, the involvement of Ca<sup>2+</sup> related kinases and RyR1 in Smad3 activation was out of scope and need to be further explored.

## 5. Conclusion

In the mouse diaphragm, MV is associated with rapid onset of oxidative modifications, occurring within 6 h. We found that mitochondrial MV-induced oxidative stress early alters RyR1 function resulting in Ca<sup>2+</sup> leakage of SR into the cytosol. We propose that this event can account for the muscular weakness induced by MV through alterations of excitation-contraction coupling. We also propose that with a longer period of MV (12 h) this impairment in Ca<sup>2+</sup> homeostasis contributes to a secondary activation of the protease Ca<sup>2+</sup> dependent system linked to disassembly of actomyosin complexes and atrophy. Several of the mechanistic pathways described above and implicated in the development of VIDD are illustrated in Fig. 6.

The results showing that treatment with SS31, a mitochondria-targeted antioxidant, prevented all of these changes, emphasize the central role of this organelle in mediating Ca<sup>2+</sup> homeostasis impairment induced by MV that fully unloads the diaphragm.

## Acknowledgment

The author would like to thank Nathalie Saint from INSERM U1046, Beatrice Chabi, Anne Bonnieu and Gilles Fouret from INRA for constructive advices in regard to the experiments performed in this manuscript.

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