Dexmedetomidine Impairs Diaphragm Function and Increases Oxidative Stress but Does Not Aggravate Diaphragmatic Atrophy in Mechanically Ventilated Rats

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ABSTRACT

Background: Anesthetics in ventilated patients are critical as any cofactor hampering diaphragmatic function may have a negative impact on the weaning progress and therefore on patients' mortality. Dexmedetomidine may display antioxidant and antiproteolytic properties, but it also reduced glucose uptake by the muscle, which may impair diaphragm force production. This study tested the hypothesis that dexmedetomidine could inhibit ventilator-induced diaphragmatic dysfunction.

Methods: Twenty-four rats were separated into three groups (n = 8/group). Two groups were mechanically ventilated during either dexmedetomidine or pentobarbital exposure for 24 h, referred to as interventional groups. A third group of directly euthanized rats served as control. Force generation, fiber dimensions, proteolysis markers, protein oxidation and lipid peroxidation, calcium homeostasis markers, and glucose transporter–4 (Glut-4) translocation were measured in the diaphragm.

Results: Diaphragm force, corrected for cross-sectional area, was significantly decreased in both interventional groups compared to controls and was significantly lower with dexmedetomidine compared to pentobarbital (*e.g.*, 100 Hz: –18%, $P < 0.0001$). In contrast to pentobarbital, dexmedetomidine did not lead to diaphragmatic atrophy, but it induced more protein oxidation (200% *vs.* 73% in pentobarbital, $P = 0.0015$), induced less upregulation of muscle atrophy F-box (149% *vs.* 374% in pentobarbital, $P < 0.001$) and impaired Glut-4 translocation (–73%, $P < 0.0005$). It activated autophagy, the calcium-dependent proteases, and caused lipid peroxidation similarly to pentobarbital.

Conclusions: Twenty-four hours of mechanical ventilation during dexmedetomidine sedation led to a worsening of ventilation-induced diaphragm dysfunction, possibly through impaired Glut-4 translocation. Although dexmedetomidine prevented diaphragmatic fiber atrophy, it did not inhibit oxidative stress and activation of the proteolytic pathways. **(ANESTHESIOLOGY 2018; XXX:00-00)**

VENTILATOR-INDUCED diaphragmatic dysfunction (VIDD), which is defined as a time-dependent decrease of diaphragm strength after initiation of mechanical ventilation (MV), has been investigated for nearly two decades.[1] Studies in different species[2–4] and humans[5] revealed a loss of function and/or atrophy of the diaphragm with enhanced proteolysis,[6,7] autophagy,[8] and downregulation of protein synthesis[9] linked to oxidative stress.[10–12] Importantly, these effects develop immediately after the onset of MV.[13,14] VIDD may thus contribute to intensive care unit (ICU)–acquired diaphragm weakness, but other factors such as systemic inflammation and sepsis are playing an important role, too.[15–17] Since diaphragm dysfunction seems to influence the clinical outcome of mechanically ventilated patients with respiratory failure, especially in ICUs,[18–21] the importance of VIDD should not be neglected.

What We Already Know about This Topic

- Dexmedetomidine is a commonly used sedative in ventilated patents in intensive care units. It has complex effects on muscle metabolism and its effects on the diaphragm are poorly understood.

What This Article Tells Us That Is New

- After 24 h sedation in rats, dexmedetomidine (*vs.* pentobarbital) increased protein oxidation and impaired glucose transport and contractile force in the diaphragm, but did not cause atrophy. The impact on ventilator-induced diaphragmatic dysfunction and weaning requires study.

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Various drugs may further affect VIDD-relevant pathomechanisms; in particular, common anesthetics have been proven to hamper diaphragmatic function in animal models of MV.[22,23] Importantly, in ICU patients, Hermans *et al.*[24] showed a link between the degree of diaphragmatic contractile dysfunction and several drugs such as analgesics and sedatives, although these authors could not exclude interference of other cofactors.

However, any additional cofactor may hamper diaphragmatic function and may have a negative impact on the weaning progress. It is therefore essential to ensure that the anesthetic drugs used in the ICU would not contribute to diaphragm dysfunction. The central α_2-agonist dexmedetomidine is an anesthetic that has been established in the United States for decades due to its good controllability of sedation depth.[25] It may represent an attractive and potential agent to impede VIDD considering its protective role as antioxidant and anticatabolic agent. Dexmedetomidine reduced oxidative stress and caspase-3 expression caused by ischemia-reperfusion injury in skeletal muscle[26,27] and attenuated muscle wasting associated with sepsis through a reduction in proteolytic markers such as Muscle Ring-finger protein-1 and muscle atrophy F-box.[28] Contrarily, dexmedetomidine can also reduce glucose uptake by skeletal muscle *in vivo*,[29] which may exacerbate VIDD. This study was conducted to test the hypothesis that dexmedetomidine is able to prevent VIDD by preservation of diaphragmatic function through reduction of oxidative stress and inhibition of the proteolytic pathways.

Materials and Methods

The study was approved by the appropriate governmental institution (Landesamt für Natur-, Umwelt- und Verbraucherschutz, North Rhine-Westphalia, Germany; reference No. AZ 84-02.04.2014.A281) and conducted in accordance with the principles for care and use of animals based on the Helsinki Declaration.

Ventilation and Anesthetic Model

Healthy 3-month-old, 300-g, female Sprague-Dawley rats (n = 24) were separated into three groups. Animals were not randomized to the groups, and the experimenters could not be blinded to the experimental conditions because preparation of the sedatives was different. Two groups were mechanically ventilated during either dexmedetomidine (D-CMV; n = 8) or pentobarbital exposure (P-CMV; n = 8) for 24 h, referred to as interventional groups. A third group of directly euthanized rats (control group; n = 8) served as control.

Anesthesia was initiated by intraperitoneal injection of 60 mg pentobarbital · kg^{-1} · body $weight^{-1}$ in all groups and sustained in the interventional groups continuously for 24 h by continuous intravenous infusion of either P-CMV or D-CMV through the jugular vein to suppress spontaneous breathing. To ensure an adequate level sedation, the animals were closely monitored by testing pinch tails and foot reflex, and sedation was also adapted to exclude diaphragm contraction during the experiment (Supplemental Digital Content 1, http://links.lww.com/ALN/B606, and Supplemental Digital Content 2, http://links.lww.com/ALN/B607).

After anesthesia induction, all animals were tracheotomized. In P-CMV and D-CMV, volume-controlled ventilation was performed with a respiratory rate of 60 breaths/min, a tidal volume of 6 to 7 ml · kg^{-1} · $bodyweight^{-1}$ and inspiration/expiration at a ratio of 1:1 to prevent hypercapnia, and a positive end-expiratory pressure of 3 to 5 cm H_2O (Evita 2, Draeger, Germany). The control group was also mechanically ventilated for approximately 5 min before removing the diaphragm to prevent hypercapnia and hypoxia.

All D-CMV and P-CMV animals were monitored continuously using electrocardiography, as well as invasive mean arterial blood pressure and rectal temperature measurements. Body temperature was maintained at 37°C. Normovolemia was ensured by continuous fluid application of 2 ml/h *via* a central venous catheter. Every 3 h, arterial blood samples were taken from the carotid artery. The bladder was expressed manually every hour. The diaphragm was removed after 24 h under sustainment of respiration and circulation, and then all animals were euthanized using an overdose of pentobarbital.

In Vitro *Diaphragm Contractility Analysis and Histology*

The diaphragmatic muscular force, corrected for cross-sectional areas, was assessed *in vitro* by stepwise stimulation from 1 to 160 Hz according to our established protocol.[23,30] Embedded and frozen diaphragmatic tissue (TekTM, Sakura, The Netherlands) was used for the histologic assessment of muscle fibers. Diaphragmatic muscle sections (7 μm) were stained using primary antibodies against type I muscle fibers (No. A4.480, Development Studies Hybredoma Bank, USA), type IIA muscle fibers (No. SC71, Development Studies Hybredoma Bank), dystrophin (Thermo Fisher Scientific, USA), and the secondary antibodies Rhodamine Red, Alexa Fluor 350, and Alexa Fluor 488 (all Invitrogen, Germany). Muscle fiber cross-sectional areas were examined (200 fibers from each animal) using the software ImageJ (v1.46k; National Institutes of Health, USA). Hematoxylin and eosin staining was also performed to assess potential structural alterations.

Protein Extraction for Electrophoresis and Western Blotting

Diaphragm tissue samples were homogenized 1:10 (weight/volume) in 5 mmol Tris·HCl and 5 mmol EDTA buffer (pH at 7.5) containing a protease inhibitor cocktail (Roche, Switzerland) and centrifuged at 1,500*g* for 10 min at 4°C. After collection of the resulting supernatant, diaphragmatic protein content was assessed by the method of Bradford[31] (Bio-Rad Laboratories Inc., USA). For cellular fractionation, diaphragm samples were homogenized in ice-cold lysis buffer (2 mM EDTA, 10 mM EGTA, 0.25 M sucrose, protease

inhibitor cocktail, and 20 mM Tris HCl at pH 7.5) and centrifuged at 900*g* for 10 min at 4°C. The obtained supernatant was centrifuged at 100,000*g* for 30 min at 4°C. The supernatant was used as a cytosolic lysate, and the pellet was carefully redissolved in radioimmunoprecipitation assay buffer lysis buffer and centrifuged at 100,000*g* for 30 min (4°C) to obtain the crude membrane fraction. Diaphragmatic protein concentration was then determined with the Pierce BSA (Thermo Fisher Scientific) protein assay kit.

For all Western blots, total proteins were separated on polyacrylamide gel and transferred onto polyvinylidene fluoride membranes. Blots were incubated overnight at 4°C with a primary antibody and subsequently with the appropriate secondary antibody for 1 h at room temperature. Proteins were detected with Chemiluminescent Peroxidase Substrate (Sigma-Aldrich, Belgium), imaged with the Proxima 2850T imaging system (Isogen Life Technologies, The Netherlands) and analyzed using the TotalLab 1D software (Isogen Life Technologies).

Assessment of Proteolytic Markers

Autophagy. Autophagosome formation was assessed by measuring the conversion of LC3B-I to LC3B-II *via* immunoblotting (4108S, Cell Signaling/Bioke, The Netherlands). Data were expressed as the LC3B-II/LC3B-I ratio.

Calcium-dependent Proteases. *In vivo* calpain-1 and caspase-3 activities were indirectly assessed by measuring cleavage of αII-spectrin (α-Fodrin FG6090, Enzo Life Science, Belgium), a specific substrate for calpain and caspase-3. Activities of calpain and caspase-3 were measured as the ratio between the densitometric values of their breakdown products to intact αII-spectrin.[12] The cleavage product of intact αII-spectrin by calpain gives a band at 150 kDa and a band at 120 kDa when cleaved by caspase-3, while intact αII-spectrin is detected at 260 kDa.

Ubiquitin Proteasome System. The E3-ligases MAFbx (sc-166806, Santa Cruz, bio connect, The Netherlands) and MuRF1 (sc-514767, Santa Cruz, bio connect) were assessed as markers of the ubiquitin proteasome system. Blots were also probed with an anti–α-tubulin antibody (T6074, Sigma-Aldrich), and data were expressed as a ratio between densitometric values of MAFbx (or MuRF1) to α-tubulin (T6074, Sigma-Aldrich).

Regulators of Autophagy. Because the autophagy-lysosome pathway and the ubiquitin proteasome system are controlled by the protein kinase B (Akt) and the forkhead box O (FoXO) transcription factors, we measured total and phosphorylated Akt (p-Akt) and FoXO1. We first assessed the phosphorylated proteins (p-Akt, 4060S, or phosphorylated FoXO1, 9461S, Bioke, The Netherlands). After detection, the antibodies were stripped from the membrane (Restore Western Blot Stripping Buffer, Thermo Fisher Scientific), and the total Akt (9272S) and total FoXO1 (2880S) proteins were detected. The blots were subsequently probed with an anti–α-tubulin (Akt and p-Akt) or an anti–β-tubulin (FoXO1 and phosphorylated FoXO1) antibody to normalize the data.

Assessment of Oxidative Stress. Protein oxidation was analyzed by detection of protein carbonyls using a detection kit (Oxyblot, Merck Millipore, Germany). Oxyblots were incubated with the anti-Dinitrophenol primary and its secondary antibody, as recommended by the manufacturer's instructions. To quantify the amount of oxidation, we defined the oxidative index as the ratio between densitometric values of the oxidized protein bands and β-tubulin.[32]

Diaphragmatic 4-hydroxynonenal (4-HNE) was used as a marker of lipid peroxidation. Polyclonal anti–4-HNE antibody (No. MAB3249, R&D Systems, United Kingdom) was used as primary antibody and polyclonal rabbit antimouse (No. P0260, Agilent, Belgium) as secondary antibody. Data were expressed as a ratio between densitometric values of 4-HNE to Ponceau S.

Assessment of Calcium Homeostasis

Impaired calcium homeostasis associated with a loss of diaphragm force but with no atrophy has been reported in the diaphragm during MV.[33,34] We therefore assessed the expression of the ryanodine receptor and the sarco(endo)plasmic calcium adenosine triphosphatase (SERCA) pumps in our model.

Ryanodine Receptor. Because the ryanodine receptor is a large protein, transfer to a polyvinylidene fluoride membrane was run overnight, and afterward the blot was first probed with an antiryanodine receptor antibody (ab6828, Abcam, United Kingdom) and then with an anti–β-tubulin antibody (T5201, Sigma-Aldrich). Data of ryanodine receptor were normalized with the data of β-tubulin.

SERCA Pumps. Blots were probed with an antibody against SERCA1 (ab-2819, Abcam) or SERCA2 (ab-2861, Abcam) and with α-tubulin (T6074, Sigma-Aldrich). Data were normalized with α-tubulin and expressed as a ratio between SERCA1 (or SERCA2) and α-tubulin (T6074, Sigma-Aldrich).

Assessment of Glucose Transporter–4 Translocation. Glucose transporter–4 (Glut-4) translocation was measured by assessing Glut-4 expression (PA1-1065, Thermo Fisher Scientific) in the cytosol and in the membrane fraction. Data were normalized with α-tubulin data (T6074, Sigma-Aldrich).

Statistical Analysis

In this pilot study setup, a sample size of eight was chosen (1) based on our experience with this animal model,[35,36] (2) taking into account a mortality rate of 10% after 24 h of MV, and (3) with the goal to reach a significant decrease in diaphragm tetanic force of 33% after 24 h of MV compared to controls. Population distribution was assessed with the Shapiro-Wilk test. As this test showed normal distribution of data, comparisons between groups for each dependent variable were made by a one-way ANOVA. A Tukey *post hoc* test was used for pairwise comparisons between all groups and for the force frequency measurements using a two-way repeated-measures ANOVA. No data sets were excluded due

to lacunarity. Data are shown as means ± SD. All statistical tests are two-tailed; significance was established at $P < 0.05$ (GraphPad Prism 6.0, USA).

Results

Physiologic Variables during the Experiment

Mean arterial blood pressure was stable throughout the entire experimental period. Mean arterial blood pressures were 86 ± 13 mmHg (P-CMV) and 96 ± 22 mmHg (D-CMV) before euthanization (Supplemental Digital Content 3, http://links.lww.com/ALN/B608). Data of blood gases and pH did not differ between all interventional groups (Supplemental Digital Content 4, http://links.lww.com/ALN/B609).

Diaphragm Function

The diaphragm force frequency curve, corrected for cross-sectional areas, was shifted downward significantly at all stimulation frequencies in the P-CMV and even more so in the D-CMV groups compared to the control group (fig. 1). Diaphragm force loss in the D-CMV group averaged 54% (range, 41 to 66%) compared to the control group. Importantly, the force was significantly lower in the D-CMV group compared to the P-CMV at stimulation frequencies of 30 Hz (–38%, $P < 0.01$), 60 Hz (–29%, $P < 0.005$), and 100 Hz (–18%, $P < 0.0001$) with an average value of 28% (fig. 1).

Diaphragm Histology

Hematoxylin and eosin staining of the diaphragm did not show any structural alterations or signs of injury with either sedatives (fig. 2). Histologic planimetry of diaphragmatic fiber dimensions revealed a significant atrophy of type I fibers (–23%, $P = 0.0101$), type IIa fibers (–24%, $P = 0.0301$), and type IIb/x fibers (–34%, $P = 0.0089$) of P-CMV animals compared to the control group, whereas no such atrophy was observed for the D-CMV animals (fig. 3).

Assessment of Proteolytic Markers

Autophagy. Autophagic activity, measured as the ratio of LC3B-II/LC3B-I, was significantly and similarly increased in P-CMV (137%, $P = 0.0032$) and D-CMV (137%, $P = 0.0055$) compared to the control group (fig. 4).

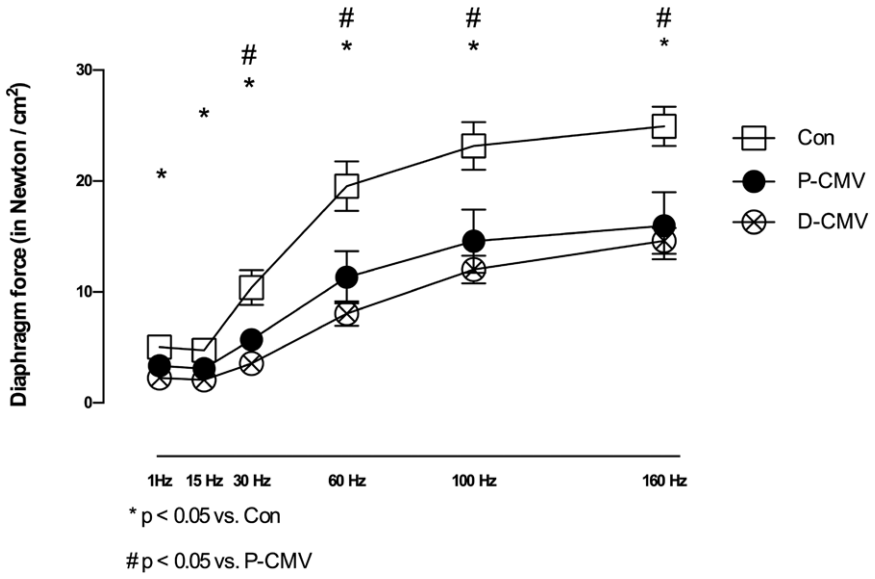

Fig. 1. Force frequency relationship of the diaphragm *in vitro* in N/cm² at 1 Hz, 15 Hz, 30 Hz, 60 Hz, 100 Hz, and maximal diaphragm force at 160 Hz. *White square*, control group (Con); *black circle*, interventional group treated with 24 h of mechanical ventilation (MV) during pentobarbital exposure (P-CMV); *crossed circle*: interventional group treated with 24 h of MV during dexmedetomidine exposure (D-CMV). Values are displayed as means ± SD.

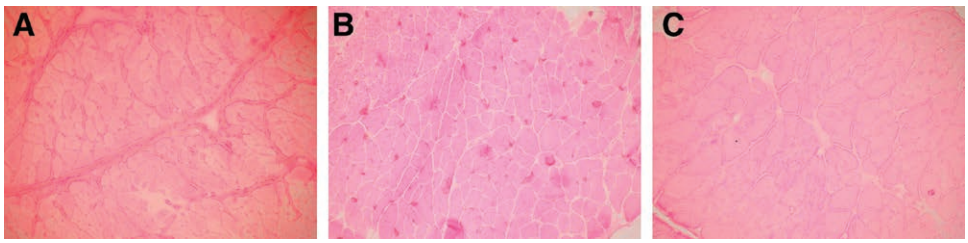

Fig. 2. Representative examples of hematoxylin and eosin staining of diaphragm from (*A*) control, (*B*) interventional group treated with 24 h of mechanical ventilation (MV) during pentobarbital exposure, and (*C*) interventional group treated with 24 h of MV during dexmedetomidine exposure.

Calcium-dependent Proteases. Calpain activity was significantly increased in P-CMV (45%, P = 0.483) and D-CMV (61%, P = 0.0070) compared to the control group (fig. 5, A and B). For caspase-3 activity, no significant differences were found between all groups (fig. 5, A and C).

Ubiquitin Proteasome System. While diaphragmatic MuRF1 expression was unaltered with either sedative (Supplemental Digital Content 5, http://links.lww.com/ALN/B610), there was a significant increase in MAFbx expression in D-CMV (149%, P < 0.01) and even more so in P-CMV (374%, P < 0.01) compared to the control group (fig. 6). Importantly, MAFbx upregulation was significantly less in D-CMV compared to P-CMV (P < 0.001) (fig. 6).

Regulators of Autophagy and Proteasome System

Total Akt levels were significantly and similarly decreased in P-CMV (–13%, P < 0.05) and D-CMV (–17%, P < 0.01) compared to the control group (fig. 7, A and B). Likewise, phosphorylated Akt expression was significantly lower with both sedatives compared to the control group

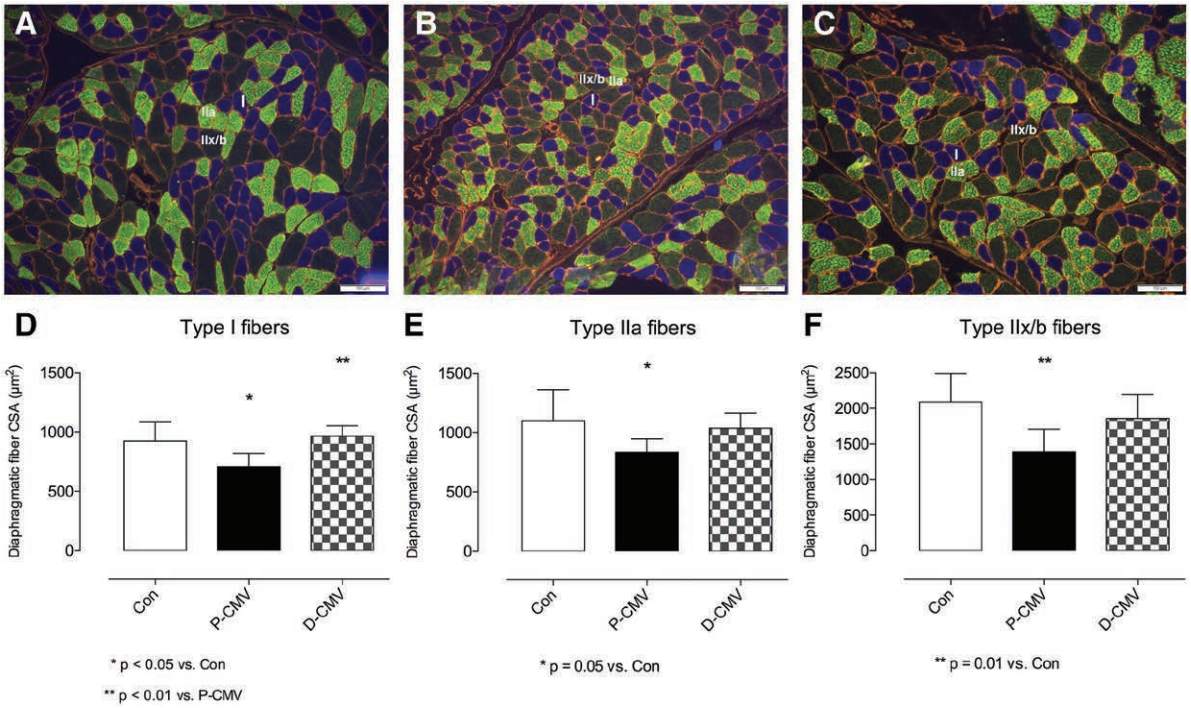

Fig. 3. Diaphragmatic fiber dimensions. Representative examples of immunostaining of the diaphragm muscle section in (*A*) a control group (Con) animal, (*B*) an animal during pentobarbital exposure (P-CMV), and (*C*) an animal during dexmedetomidine exposure (D-CMV). Cross-sectional areas (CSAs) in µm^2 of *(D)* type I, *(E)* IIa, and *(F)* IIx/b fibers in Con, 24 h of mechanical ventilation (MV) P-CMV, and 24 h of MV D-CMV. Values are displayed as means ± SD.

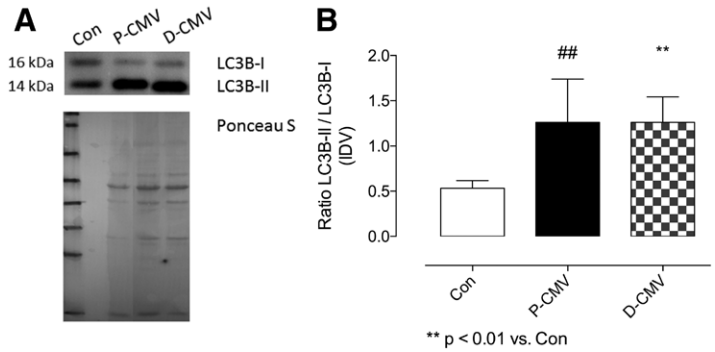

Fig. 4. Autophagosome formation assessed in the diaphragm by measuring the conversion of LC3B-I to LC3B-II. (*A*) Representative immunoblots of LC3B-I and LC3B-II and Ponceau S in each group. (*B*) Diaphragm levels of activated LC3B expressed as the ratio of LC3B-II/LC3B-I in the control group (Con), and interventional group treated with 24 h of mechanical ventilation (MV) during pentobarbital exposure (P-CMV) or during dexmedetomidine exposure (D-CMV). Values are displayed as means ± SD. ## P < 0.01 vs. Con. IDV = integrated density value.

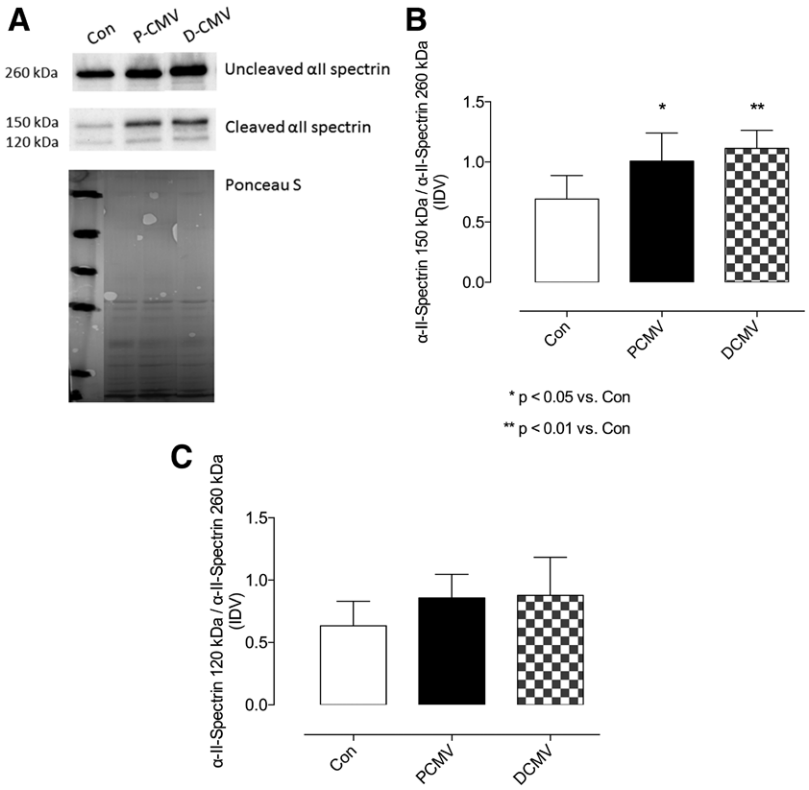

Fig. 5. Assessment of calcium-dependent proteases in the diaphragm of the control group (Con) and interventional group treated with 24 h of mechanical ventilation (MV) during pentobarbital exposure (P-CMV) or during dexmedetomidine exposure (D-CMV). (*A*) Representative immunoblots of uncleaved and cleaved αII spectrin and Ponceau S for each group. (*B*) Indirect assessment of calpain-1 activity as marker of protein breakdown in the diaphragm, measured as ratio of the 150 kDa breakdown product of α-II-spectrin to the uncleaved (260 kDa). (*C*) Indirect assessment of caspase activity as marker of protein breakdown in the diaphragm, measured as ratio of the 120 kDa breakdown product of α-II-spectrin to the uncleaved (260 kDa). Values are displayed as means ± SD. All data presented as integrated density value (IDV).

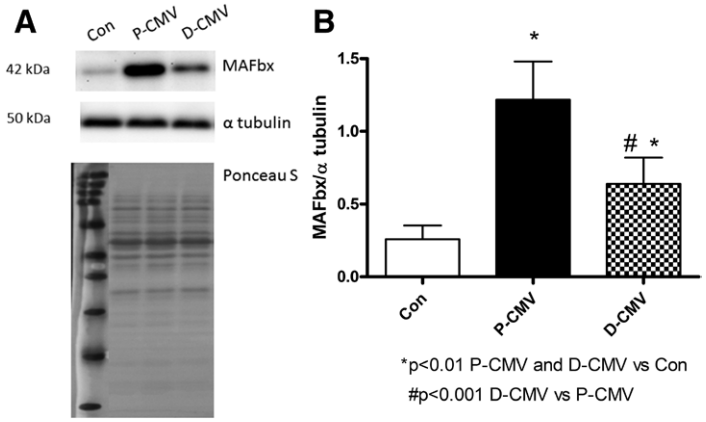

Fig. 6. Diaphragm muscle atrophy F-box (MAFbx) protein expression in the control group (Con) and interventional group treated with 24 h of mechanical ventilation (MV) during pentobarbital exposure (P-CMV) or during dexmedetomidine exposure (D-CMV). (*A*) Representative immunoblots of MAFbx and α-tubulin and Ponceau S in each group. (*B*) MAFbx data normalized to α-tubulin. Values are displayed as means ± SD. All data presented as integrated density value.

(P-CMV, –21%, and D-CMV, –17% *vs.* the control group, $P < 0.05$; fig. 7, A and B). While FoXO1 expression significantly increased by 27% in P-CMV compared to the control group ($P < 0.01$), it remained unchanged in D-CMV (fig. 7, C and D). Conversely, phosphorylated FoXO1 levels decreased significantly with both sedatives compared to the control group (P-CMV, –34% and D-CMV, –21% *vs.* the control group, $P < 0.01$; fig. 7), and this decrease was significantly more severe in P-CMV (–17%, $P < 0.001$) compared to D-CMV (fig. 7D).

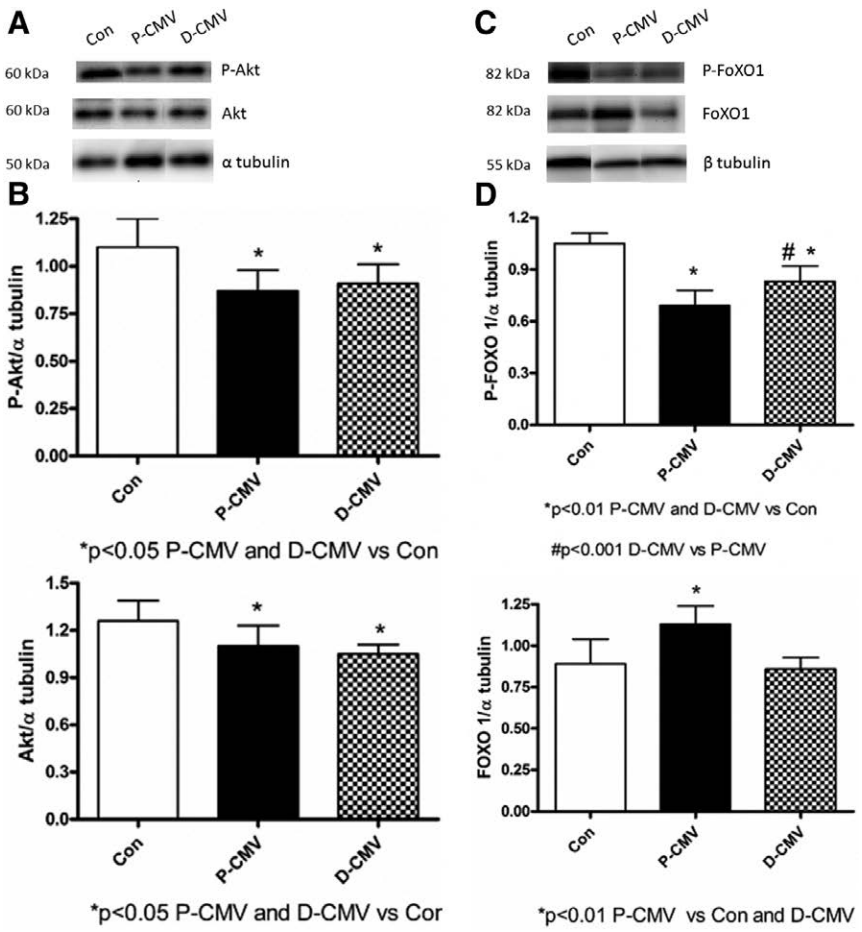

Fig. 7. Diaphragmatic protein kinase B (Akt)/forkhead box O–1 (FoXO1) measures in the control group (Con) and interventional group treated with 24 h of mechanical ventilation (MV) during pentobarbital exposure (P-CMV) or during dexmedetomidine exposure (D-CMV). (*A*) Representative immunoblots by group for phosphorylated Akt (p-Akt), total Akt, α-tubulin. (*B*) Levels of p-Akt and total Akt normalized to α-tubulin. (*C*) Representative immunoblots by group for phosphorylated FoXO1 (p-FoXO1), total FoXO1, and β-tubulin. (*D*) Levels of p-FoXO1 and total FoXO1 normalized by β-tubulin. Values are displayed as means ± SD. All data presented as integrated density value.

Oxidative Stress

Lipid peroxidation indicated that diaphragmatic 4-HNE levels were significantly increased to the same extent in P-CMV (38%, $P = 0.0027$) and D-CMV (40%, $P = 0.0019$) compared to the control group (fig. 8). Protein oxidation significantly increased in D-CMV compared to the control group (200%, $P = 0.0015$), while this increase failed to reach statistical significance in P-CMV (73%, $P = 0.2718$; fig. 9).

Calcium Homeostasis

Neither SERCA1 (Supplemental Digital Content 6A, http://links.lww.com/ALN/B611) nor SERCA2 (Supplemental Digital Content 6B, http://links.lww.com/ALN/B611) expressions were altered in the diaphragm of either P-CMV or D-CMV compared to the control group. Similarly, the ryanodine receptor expression was not altered after MV with both sedatives (Supplemental Digital Content 7, http://links.lww.com/ALN/B612).

Glut-4 Expression and Translocation

Diaphragmatic Glut-4 cytosol expression was similar in the three groups, while diaphragmatic Glut-4 membrane expression was lower in D-CMV compared to others (–74 and –72% *vs*. CON and P-CMV, respectively, $P = 0.05$; fig. 10). In addition, while diaphragmatic Glut-4 expression in the membrane fraction tended to increase in the control group (77%, $P = 0.07$) and P-CMV (86%, $P = 0.09$) compared to their respective cytosolic fraction, membrane Glut-4 expression was significantly decreased by 73% in the diaphragm of D-CMV compared to its cytosolic fraction ($P < 0.0005$; fig. 10).

Discussion

This study showed that in contrast to our hypothesis, dexmedetomidine did not protect the diaphragm from VIDD and did not act as an antioxidant and antiproteolytic agent. In fact, protein oxidation was more pronounced and upregulation of MAFbx less severe during dexmedetomidine than pentobarbital.

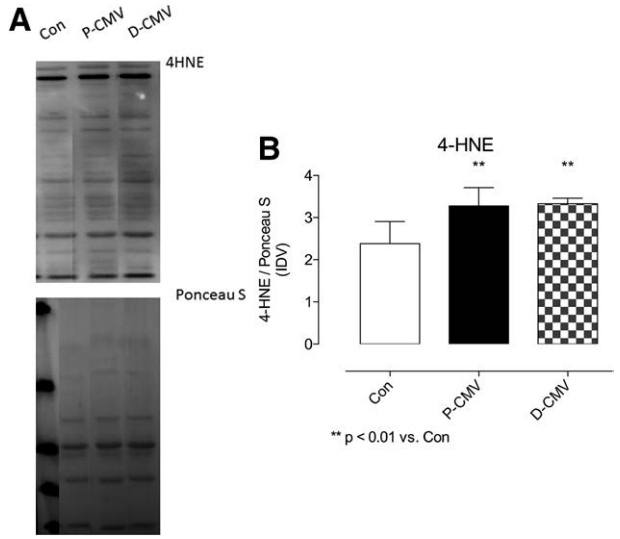

Fig. 8. Diaphragmatic lipid peroxidation in the control group (Con) and interventional group treated with 24 h of mechanical ventilation (MV) during pentobarbital exposure (P-CMV) or during dexmedetomidine exposure (D-CMV). (*A*) Representative immunoblot for 4-hydroxynonenal (4-HNE) and Ponceau S in each group. (*B*) Density of the 4-HNE bands (100, 60, 50, 37 kDa) analyzed as an indicator of lipid peroxidation. Data normalized to Ponceau S. Values are displayed as means ± SD. All data presented as integrated density value (IDV).

Importantly, dexmedetomidine prevented diaphragm fiber atrophy. Impaired Glut-4 translocation may have contributed to the loss of diaphragm force during dexmedetomidine.

Dexmedetomidine Did Not Protect the Diaphragm from VIDD

Diaphragm force loss was aggravated by dexmedetomidine, particularly at stimulation frequencies ranging from 30 to 100 Hz. The additional force deficit averaged 28% and was larger at the lower stimulation frequency (*e.g.*, 30 Hz, –38%). These data are in the range of the diaphragm force decline reported in ICU patients.[19,24] Any deficit considered at first sight as small may be sufficient to reach a certain threshold at which diaphragm force is too low to maintain spontaneous breathing and patients may subsequently fail weaning. When compared to the control group, the diaphragm force deficit during dexmedetomidine averaged 54%, a dysfunction similar to that reported during MV with propofol,[22] but worse than that caused by sevoflurane.[23] However, these data refer to *in vitro* force generation corrected for atrophy.

Dexmedetomidine Did Not Act as an Antiproteolytic Agent

Autophagy was similarly enhanced with both sedatives. Previous studies reported enhanced LC3B-II as early as 8 h[37] and up to 18 h[38] of MV with pentobarbital. Activation of autophagy is unlikely to be responsible for the additional loss of diaphragm function with dexmedetomidine.

Dexmedetomidine activated calpain to the same extent as pentobarbital. The latter is in agreement with previous reports.[14,39,40] Caspase-3 was not activated and its implication could be ruled out. Similarly, calpain activation was unlikely to explain the additional loss of diaphragm force with dexmedetomidine.

Finally, while diaphragmatic MuRF1 protein expression remained unchanged, MAFbx protein levels were enhanced, but to a lesser extent with dexmedetomidine. Upregulation of these markers has been previously reported in ventilated patients and animal models.[5,21,41,42] Our data suggest a less harmful effect of dexmedetomidine because of its reduced MAFbx activation compared to pentobarbital.

Expression of MuRF1, MAFbx, and autophagy-related genes can be blocked through phosphorylation of FoXO1 by Akt, which impedes FoXO1 transcriptional activity by hampering its translocation to the nucleus.[43–45] Our data showing decreased active Akt and enhanced FoXO1 expression during pentobarbital are consistent with the upregulation of MAFbx and autophagy-related genes. But surprisingly, with dexmedetomidine, a reduction of active Akt and phosphorylated FoXO1 was detected, fitting with the reduced MAFbx expression after dexmedetomidine exposure.

Taken together, our data indicate that dexmedetomidine did not inhibit the activation of proteolytic markers in contrast with previous studies.[27,28] The antiproteolytic properties

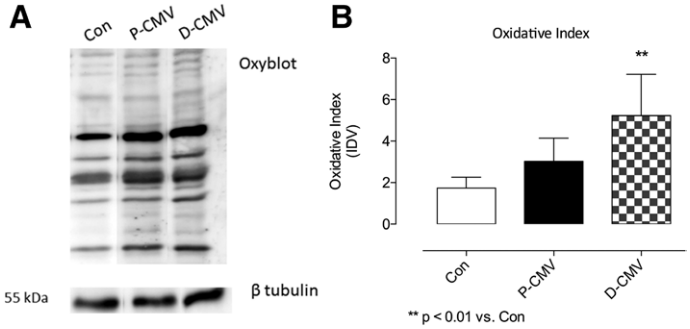

Fig. 9. Diaphragmatic protein oxidation in the control group (Con) and interventional group treated with 24 h of mechanical ventilation (MV) during pentobarbital exposure (P-CMV) or during dexmedetomidine exposure (D-CMV). (*A*) Representative immunoblot for protein oxidation (oxyblot) and β-tubulin in each group. (*B*) Oxidative index defined as the ratio between densitometric values of the oxidized proteins bands to β-tubulin. Values are displayed as means ± SD. All data presented as integrated density value (IDV).

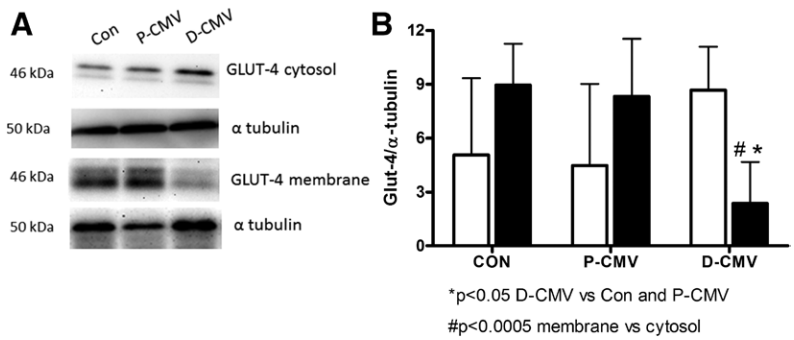

Fig. 10. Diaphragmatic glucose transporter–4 (Glut-4) expression in the control group (Con) and interventional group treated with 24 h of mechanical ventilation (MV) during pentobarbital exposure (P-CMV) or during dexmedetomidine exposure (D-CMV). (*A*) Representative immunoblot for Glut-4 in cytosol, Glut-4 in membrane, and α-tubulin in each group. (*B, open bars*) Levels of Glut-4 in the cytosol normalized to α-tubulin, and (*solid bars*) of Glut-4 in membrane normalized by α-tubulin. Values are displayed as means ± SD. All data presented as integrated density value.

of dexmedetomidine might be dependent on the model used (*e.g.*, sepsis[28] or ischemia reperfusion injury[27]) where inflammation plays a pivotal role, in contrast to muscle disuse, which is central in MV. In addition, the different approach might contribute to these discrepancies: *in vitro*[27] or *in vivo*,[28] as in our study, type of tissue studied (limb muscle[28] or retina[27] *vs.* diaphragm), and treatment regimen (single *vs.* continuous dose). Further, our data demonstrated that although dexmedetomidine activated the proteolytic pathways, the expected diaphragm fiber atrophy did not develop.

Dexmedetomidine Did Not Act as an Antioxidant Agent

Lipid peroxidation was similarly increased with both sedatives, while protein oxidation was dramatically increased during dexmedetomidine. These data indicate that dexmedetomidine did not inhibit oxidative stress as hypothesized, but on the contrary promoted protein oxidation even more than pentobarbital. These findings contradict previous reports where in different models (*e.g.*, cerebral ischemia, ischemia reperfusion injury, acute lung injury), dexmedetomidine was shown to reequilibrate the oxidant–antioxidant balance while enhancing antioxidant defense enzymes and inhibiting lipid peroxidation.[26,46–48] This has also been reported in the gastrocnemius muscle in an experimental model of ischemia reperfusion injury.[26] But in an experimental model of diet-induced obesity, dexmedetomidine failed to inhibit oxidative stress in lung tissue.[49] Our data corroborate this study and suggest that the antioxidant properties of dexmedetomidine are not constant. They also underline that dexmedetomidine by promoting protein oxidation may potentially be detrimental for the diaphragm.

Dexmedetomidine Did Not Affect Diaphragm Fiber Dimensions and Structure

While diaphragm fiber atrophy developed during pentobarbital as previously reported,[14,40,50] diaphragm fiber size was preserved during dexmedetomidine. The benefit and functional implications of this finding are highly relevant as diaphragm function depends on the size and contractility of its individual fibers. Sparing the diaphragm from atrophy is expected to result in higher total diaphragm force *in vivo*. In addition, preservation of the diaphragm from atrophy is likely to facilitate diaphragm recovery and to minimize the long-term consequences of atrophy such as impaired muscle regeneration reported in ICU survivors.[51] This protective effect of dexmedetomidine against diaphragm atrophy needs to be kept in mind.

This preservation of diaphragm fiber size is somehow inconsistent with the activation of the proteolytic pathways. It is unclear why such activation did not result in diaphragm fiber atrophy with dexmedetomidine while it did with pentobarbital. Actually, MAFbx upregulation was significantly lower with dexmedetomidine, but whether this difference could be sufficient to explain the presence of fiber atrophy with pentobarbital and its absence with dexmedetomidine is unclear and cannot conclusively be explained in this study setting. Finally, both sedatives preserved diaphragm structure, suggesting that loss of diaphragm force was not the consequence of muscle damage.

Dexmedetomidine Did Not Affect Calcium Homeostasis

Several studies have pointed out impairment of calcium homeostasis in the diaphragm during MV.[33,34,41] They reported indirect evidence for enhanced intracellular calcium levels[41] and clear rapid remodeling of the ryanodine receptor[33,34] concomitant with a reduction of diaphragm force in the absence of atrophy or histologic injury.[33,52] In the current study, neither ryanodine receptor expression nor SERCA1 and SERCA2 expression were affected by dexmedetomidine, suggesting that alterations in calcium homeostasis were not involved in the loss of diaphragm force with dexmedetomidine.

Dexmedetomidine Impaired Glut-4 Translocation

Glut-4 is the major glucose transporter isoform expressed in skeletal muscle, and its translocation from intracellular storage sites to the sarcolemma membrane upon muscle contraction is an essential step for glucose to be taken up by skeletal muscle cells. Under resting conditions, glucose

transport is believed to be the limiting step for glucose muscle uptake. Muscle exercise stimulates Glut-4 translocation,[53] and conversely, muscle inactivity downregulates Glut-4 content and reduces its translocation.[54,55] Translocation of Glut-4 supposes higher level of Glut-4 protein expression in the membrane compared to the cytosol. Pentobarbital did not affect Glut-4 translocation while membrane Glut-4 expression was reduced with dexmedetomidine, suggesting impaired Glut-4 translocation and thus reduced glucose uptake by the muscle. This is in agreement with reduced glucose reported in mice with *in vivo* administration of dexmedetomidine.[29] There is a linear relationship between muscle force generation and glucose transport,[56] which indicates that impaired glucose transport goes hand in hand with reduced muscle force production. Taken together, these data suggest that reduced Glut-4 translocation may possibly have taken part in the reduction in diaphragm force with dexmedetomidine.

Importance of the Findings

These data raise the question of how bad or good dexmedetomidine is for the diaphragm, especially after its discontinuation. Although speculative, our data likely support a more protective role of dexmedetomidine than expected. Indeed, preservation of diaphragm from atrophy is supposed to facilitate diaphragm function recovery. Similarly, the fact that Glut-4 translocation is impaired while total Glut-4 content is maintained favors a rapid reversibility, although the time needed to restore Glut-4 translocation after cessation of dexmedetomidine is not known. But the dynamic of Glut-4 translocation is a rapid phenomenon[57] that could be further stimulated by contraction with reinstitution of partial support ventilation. The latter would also help relieve oxidative stress. A combination of these effects may favor diaphragm function recovery, although this remains to be determined.

Limitations

Our findings pertain to an animal model, which might not be completely convertible to the patient. However, the translation of these models to the human pathophysiology has been proven to be based on the same pathophysiologic pathways.[1] Our data might be of value for human critical care if dexmedetomidine were used in the same way as in the current study. At the least, these data indicate that dexmedetomidine would be deleterious for diaphragm function.

This study did not investigate dose-effect relations of the sedatives, which was beyond the aim of the work. We sought to investigate the influence of the anesthetic dexmedetomidine compared to the sedative pentobarbital.

We did not include a control group breathing spontaneously for 24 h. Pentobarbital is known to have no independent impact on the development of VIDD pathophysiology.[14,39,40] For dexmedetomidine, during preliminary experiments, it appeared impossible to maintain the level of sedation deep enough to fulfill criteria of animal care and otherwise allow spontaneous breathing under reduced concentrations. This limits the transferability of our study because patients are receiving doses of dexmedetomidine not suppressing the respiratory drive: if there is a dose dependence of dexmedetomidine to diaphragm homeostasis and function, the effects described here would not account for spontaneously breathing individuals. Our results may therefore be dose dependent, but the absence of a correlation between contractile force and the cumulative dose applied suggests this is unlikely.

Conclusions

Dexmedetomidine further reduced diaphragmatic force in a 24-h rat model of MV, possibly through impaired Glut-4 translocation. It did not act as an antioxidant or anticatabolic agent, but it spared the diaphragm from atrophy. The translational impact of these findings on human intensive care needs to be addressed.

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Competing Interests

Dr. Marx received consultant fees from B. Braun AG (Melsungen, Germany), Adrenomed AG (Henningsdorf, Germany), and Covidien plc (Dublin, Ireland). The other authors declare no competing interests.

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