MECHANICAL ventilation (MV) is a life-saving intervention for patients suffering from respiratory failure or who need deep sedation. Even after 6 h of MV, a process of diaphragm atrophy is initiated with concomitant loss of diaphragm-specific force generation, collectively referred to as ventilator-induced diaphragm dysfunction (VIDD). Although not solely responsible for weaning failure, VIDD contributes to prolonged weaning, the urge for reintubation, and prolonged MV resulting in higher morbidity and mortality. Several pathways have been investigated, indicating a severe disturbance in cellular function as evidenced by increases in oxidative stress and subsequent proteolysis, autophagy, and depressed protein synthesis. It has been revealed that the process of VIDD can be aggravated by low levels of corticosteroids or neuromuscular-blocking agents. The latter findings have questioned the prolonged use of rocuronium for relaxation in the intensive care unit (ICU).

What We Already Know about This Topic
- Mechanical ventilation for 6 h or more can cause ventilator-induced diaphragm dysfunction

What This Article Tell Us That Is New
- Propofol-anesthetized rats were ventilated for 24 h or allowed to spontaneously breathe for 24 h and were compared with pentobarbital-anesthetized rats
- Diaphragm contractile deficit and atrophy were present after propofol administration in both ventilated and spontaneously breathing rats

Propofol is one of the most widely used anesthetic agents, primarily administered during general anesthesia and/or for sedation in the ICU setting. Its phenolic structure (2,6-diisopropyl phenol) is comparable with tocopherol (vitamin E) that has been demonstrated to have antioxidant capacities. A certain antioxidant capacity of propofol...
has been demonstrated in preserving cardiolipin levels of cardiac mitochondria from reactive oxygen species (ROS) interaction.8,9 Interestingly, in vessel smooth muscle cells, propofol increased ROS levels and subsequent apoptosis.10 Furthermore, in humans, Hermans et al.11 reported a relation between duration of propofol administration and the severity of diaphragm contractile dysfunction in ventilated patients. However, the study failed to isolate the effect of propofol from the detrimental effects of prolonged MV on the diaphragm.

On the basis of the latter study, the effects of propofol on different muscle types, site of action, and interaction with possible pathways of VIDD are not evident, yet. Importantly, the link between propofol use and severity of diaphragm dysfunction in ICU patients suggests that propofol sedation may have impact on patient outcome.11 We hypothesized that using propofol as sedative might reduce markers of oxidative stress and prevent fiber atrophy and contractile dysfunction in the diaphragm. We tested this with the use of our established rodent ventilation model of 24 h of MV.

Materials and Methods

Experimental Design

The study was approved by the ethical committee for animal experimentation of the Medical Faculty of the Katholieke Universiteit Leuven, Leuven, Belgium. Animal experiments, diaphragm contractile properties, and tissue sample collecting were conducted at the University of Leuven. Adult male Wistar rats were randomly assigned to one of the three groups: (1) a control group (Con, n = 8) acutely anesthetized with sodium pentobarbital, (2) a group submitted to 24 h of controlled mechanical ventilation (CMV, n = 8) receiving maintenance anesthesia with propofol 2% (AstraZeneca, Wedel, Germany), or (3) a group submitted to 24 h of spontaneous breathing (SB, n = 8) anesthetized with propofol 2%. Sodium pentobarbital dose was 60 mg/kg and the initial dose of propofol was 20 mg kg⁻¹ h⁻¹. The latter was adapted according to anesthesia depth controlled throughout the experiment by evaluating foot reflex, corneal reflex, and arterial blood pressure. The general protocol has been described previously.12 In brief, all animals were initially anesthetized with sodium pentobarbital, tracheotomized using a plastic tracheal tube, and the carotid artery was catheterized for arterial sample collection. After 24 h, segments of the costal diaphragm were removed for measurements of in vitro contractile properties as previously described.13 The remaining diaphragm samples, as well as the gastrocnemius muscle, were stored for further histological examination and muscle fiber typing and for biochemical analysis.

Finally, to rule out whether the observed effects might have been due to the carrier of propofol (Lipovenous 10%; Fresenius Kabi, Bad Homburg, Germany), a preliminary experiment was conducted in a subgroup of animals breathing spontaneously under pentobarbital anesthesia and receiving an infusion of carrier through the tail vein at a rate similar to the propofol infusion. In another preliminary experiment, we investigated the effect of pentobarbital during SB for 24 h. Because both the carrier and SB using pentobarbital did not affect diaphragm force, no further investigation was performed in these subgroups.

Muscle Histology. Diaphragm and gastrocnemius tissue was embedded in Optimal Cutting Temperature compound (Tissue Tek; Sakura Finetek Europe, Alphen aan den Rijn, The Netherlands) and frozen at −80°C in liquid nitrogen. Using a cryotome, sections of 7 μm were cut and stained using antibodies against type I fibers (A 4.840), Ila fibers (SC71; Development Studies Hybridoma Bank, University of Iowa, Iowa City, IA), and Dystrophin (Thermo Scientific, Maltham, MA) and linked with secondary antibodies. As secondary antibodies, Rhodamine red, Alexa fluor 350, and Alexa 488 (Invitrogen, Frankfurt, Germany) were used. Pictures were made using Fluorescence microscopy (Zeiss Axiovision, Jena, Germany) at 400-fold magnification. Cross-sectional area was determined using Scion image software counting approximately 250 fibers per animal (National Institutes of Health, Bethesda, MD).

Oxidative Stress Measurements and Protein Kinase B (Akt) Pathway

Protein Oxidation. The detection of protein carbonyls in the diaphragm and gastrocnemius muscle was performed using a protein oxidation detection kit (Oxylabt; Millipore, Billerica, MA). Diaphragm or gastrocnemius muscle samples were homogenized using a KPO₄ buffer, and total protein concentration was determined with the Bradford method.12 Derivatization of the samples, with dinitrophenylhydrazine, was carried out for 15 min on 10 μg of total protein following manufacturer’s instructions. The dinitrophenyl-derivatized proteins were separated on a 12% sodium dodecyl sulfate-polyacrylamide gel, transferred to a polyvinylidene difluoride membrane, and then stained with Ponceau S. After blocking, blots were incubated with the antidinitrophenyl primary and its secondary antibody, both delivered with the detection kit, and then developed using chemiluminescence (GE Healthcare, Diegem, Belgium) and analyzed with the software package (Bio 1D; Vilber Lourmat, Marne La Vallee, France) of the imaging system (Photo print, Vilber Lourmat). To quantify the amount of oxidation, we defined the oxidative index12

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Anesthesiology 2014; 120:665-72
which is the ratio between densitometric values of the oxidized proteins and the Ponceau S–stained bands.

**Lipid Peroxidation, Akt, and Forkhead Box 0-1 (FoxO-1) Measurements.** Approximately 30 mg of diaphragm tissue was homogenized on ice in 800 μl lysis buffer containing 150 mM sodium chloride, 1.0% NP-40, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, 50 mM Tris-HCl (pH 7.6; all from Sigma Aldrich, Hamburg, Germany), and Protease inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany), using 2-ml Potter-S homogenization cylinders (Sartorius, Goettingen, Germany). Homogenates were centrifuged through Qiagen-shredder columns (Qiagen, Hilden, Germany) at 390g for 2 min and the supernatant was used for the determination of protein concentrations, using a DC-Protein Assay Kit (Bio-Rad Laboratories, München, Germany). Samples were boiled for 5 min after addition of dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories). After the semi-dry blotting procedure (60 min, 25 V), the membrane was incubated for 1 h at room temperature in 5% bovine serum albumin-blocking solution (Albumin fraction V; ROTH, Karlsruhe, Germany), followed by overnight incubation on a shaker at 4°C with specific antibodies against 4-hydroxynonenal (Abcam, Cambridge, United Kingdom), Akt (Cell signaling, Danvers, MA), FoxO-1 (Cell signaling), and glyceraldehyde-dehyd-3-phosphat-dehydrogenase as a loading control (Cell signaling). Overnight incubation was followed by repeated washing steps (3 × 5 min in Tris-buffered saline buffer containing 1% Tween20; Sigma Aldrich), then the membrane was incubated for 1 h at room temperature with horseradish-peroxidase–conjugated goat anti-rabbit antibody (#7074; Cell signaling). The final reaction was visualized using enhanced chemiluminescence (WEST-ZOL Plus Western Blot detection system; iNtRON Biotechnology, Sungnam, Korea) and a detection system (BioDocAnalyze Live; Biometra, Goettingen, Germany). Images were taken and densitometrically analyzed with the software package (Bio 1D; Vilber Lourmat) of the imaging system (Photo print; Vilber Lourmat). Calpain and caspase-3 activity are expressed as the ratio between the densitometric values of their specific breakdown products to the intact αII-spectrin.

**Statistical Analysis**

Population distribution was assessed with Shapiro–Wilk test. Comparisons for each dependent variable (muscle fiber size, 4-hydroxynonenal and Akt, FoxO-1) were made by a one-way ANOVA using Prism 6, GraphPad Software Inc. (La Jolla, CA), whereas for the force frequency measurements, a two-way repeated-measures ANOVA was performed with the SAS software (SAS 9.3; SAS Institute, Cary, NC). When appropriate, significant group differences were evaluated using a Tukey post hoc test. Significance was established at a P value less than 0.05 using two-sided P values. Data are shown as means ± 95% CI. The sample number for each measurement is indicated in the figure legends.

**Results**

**Systemic and Biological Response**

In the CMV group, one animal died during the experiment so that the final number of animals for this group was seven. Arterial blood pressure and blood gas data were similar between the different groups. Blood pressures were 108 ± 27 mmHg (CMV) and 102 ± 9 mmHg (SB) before sacrifice, the PaO2 at the end of the experiment was 114 ± 25 (CMV) and 99 ± 35 mmHg (SB), the PaCO2 was 40 ± 11 (CMV) and 39 ± 10 mmHg (SB), with a pH of 7.33 ± 0.1 (CMV) and 7.31 ± 0.1 (SB). The CMV and SB groups received 21.7 ± 3 and 18.8 ± 1 mg kg⁻¹ h⁻¹ of propofol, respectively.

**Diaphragm In Vitro Contractile Properties**

In the CMV and SB groups, the force frequency curve was shifted downward significantly at all stimulation frequencies greater than 50 Hz compared with Con (fig. 1). This reduction in force was similar for both the CMV and SB groups. Tetanic force was significantly decreased by 34% in the CMV animals and 31% (P < 0.001) in the SB group, when compared with Con.

**Muscle Histology**

A generalized and significant fiber atrophy was found in the diaphragm of the CMV group compared with Con (fig. 2). Similarly, generalized fiber atrophy was also present in the diaphragm of the CMV group compared with Con (fig. 2). In the gastrocnemius muscle, fiber size was not changed compared with Con.

**Protein Oxidation**

The oxidative index in the diaphragm was significantly increased by 51% (P < 0.01) in the CMV group compared with Con.
Propofol Induces Diaphragm Dysfunction and Atrophy

Propofol Induces Diaphragm Dysfunction and Atrophy with Con while it remained unchanged in the SB group (fig. 3A). In the gastrocnemius, there were no differences in protein oxidation between the three groups. Compared with Con, lipid peroxidation/protein adducts (4-hydroxynonenal) was significantly increased in the diaphragm in the CMV ($P = 0.04$), but not in the SB group (fig. 3B).

Calpain and Caspase-3 Activity

Calpain activity was significantly increased in the CMV group (63%) compared with Con ($P = 0.005$) (fig. 4), whereas it was unchanged in the SB group. Caspase-3 activity was not different between the three groups.

Discussion

Overview of Principal Findings

These experiments provide novel insights into the use of one of the major anesthetic drugs used for both short- and long-term sedation. Importantly, in contrast to previous
work regarding SB and diaphragm atrophy and weakness, both SB and MV groups demonstrated contractile deficits and fiber atrophy when sedated with propofol. Interestingly, markers of oxidative stress and proteolytic activity were only increased in the MV group, but not in SB animals. A brief discussion of these points follows.

**Diaphragm Dysfunction and Atrophy Occur in Both Ventilated and SB Animals under Propofol Sedation**

Animals submitted to CMV under propofol sedation developed VIDD and atrophy after 24 h. These results are in line with several publications in animals sedated with pentobarbital, showing loss of diaphragm contractile function and fiber atrophy after CMV.\(^{15-17}\) Surprisingly, our findings reveal a negative impact of propofol on the diaphragm in SB animals, which is in contrast to the data reported in SB animals sedated with sodium pentobarbital.\(^{15,16,18}\) Importantly, our findings reveal that diaphragm weakness and atrophy are related to the use of propofol solution as it is present in both SB animals and animals under MV. Although it is tempting to attribute this effect to the dose of propofol used in the current study, several arguments indicate that the dose is unlikely to explain those data. First, although this dose seems high when compared with human situations, this is in fact not the case. Indeed, the dose of propofol was pharmacologically scaled according to the higher metabolic function of rats; actually, this dose corresponds to a propofol dose of approximately 3.5 mg kg\(^{-1}\) h\(^{-1}\) in humans.\(^{19}\)

**Fig. 4.** Calpain activity index (A) and caspase 3 (B) as markers of protein breakdown. White bar: control, light grey: controlled mechanical ventilation (CMV), dark grey: spontaneous breathing (SB). CMV: \(n = 5\); Con = control: \(n = 7\); SB: \(n = 8\). \(^*P < 0.05\) versus others.

**Fig. 5.** Ratio of phosphorylated/dephosphorylated AKT (A) and FoxO-1 (B). White bar: control, light grey: controlled mechanical ventilation (CMV), dark grey: spontaneous breathing (SB). CMV (A: \(n = 6\); B: \(n = 7\)), Con = control (A: \(n = 8\), B: \(n = 8\)), SB (A: \(n = 7\), B: \(n = 7\)). \(^*P < 0.05\) versus others. GAPDH = glycerinaldehyde-3-phosphat-dehydrogenase; IDV = integrated density value.
Propofol Induces Diaphragm Dysfunction and Atrophy

Obviously, this dose is below that needed for anesthesia in humans and fits within the ranges for an ICU sedative regimen. Second, none of our animals did show any signs of propofol infusion syndrome that would be expected with high dose of propofol. Third, propofol dose up to 40 mg kg\(^{-1}\) h\(^{-1}\) has been used by others in rats\(^{20}\), indicating that our dose of 20 mg kg\(^{-1}\) h\(^{-1}\) is not unexpected in rats. Next to the dose, the role of the lipid carrier of propofol needs to be addressed especially because lipid accumulation as a result of metabolic oversupply has been recently reported in the diaphragm after MV\(^{21}\). However, our preliminary experiment showing that the infusion of the lipid carrier was not associated with any effects on the diaphragm excludes a direct action of the lipids on diaphragm force and atrophy.

Of note, we observed a contractile deficit at all stimulation frequencies and a profound atrophy of all the diaphragm fibers (approximately a 30% reduction) in the SB and the CMV groups, which were nearly equal. Interestingly, these findings were absent in gastrocnemius muscle tissue. Recent publications have demonstrated that there is no effect (yet) on gastrocnemius\(^{22}\) during 24 h of disuse, but longer time spans of disuse are needed to show equal atrophy in gastrocnemius muscle.\(^{23}\) Therefore, we can state that propofol depresses diaphragm function to the same extent as if the animal had been mechanically ventilated.

Propofol Interacts with Pathways of VIDD

Due to its phenolic structure, propofol has been linked to ROS scavenging properties in different clinical settings, such as sepsis\(^{8}\) or during ischemia–reperfusion injury,\(^{24}\) through inhibition of the opening of mitochondrial transition pores.\(^{25}\) Contrarily, in smooth muscle cells, propofol has led to an increase in oxidative stress.\(^{10}\) Interestingly, in experiments under pentobarbital anesthesia, ROS production has been revealed as one major contributor to VIDD, with the mitochondria as the major source of oxidative stress.\(^{26}\) Currently, CMV under propofol sedation induced an increase in oxidative stress and downstream protease action. These proteases are activated with increased oxidative stress\(^{27}\) and are linked in a cross-talk–like manner.\(^{28}\) Actually, our data showing increased protein and lipid oxidation and increased cleavage products of calpain after CMV agree with previous work under pentobarbital anesthesia. These data indicate that propofol did not protect the diaphragm from CMV-induced oxidative stress, or at least its ROS scavenging action was insufficient compared with the amount of oxidative stress present. Notably, oxidative stress and increased downstream proteases such as calpain and caspase-3 were not observed in our spontaneously breathing animals. This is similar to the data reported under pentobarbital anesthesia. Interestingly, the absence of caspase-3 activation in our CMV group is an important observation knowing that caspase-3 is one the major active proteases during VIDD.\(^{3}\) These data suggest that caspase-3 activation caused by CMV is hampered in the presence of propofol. Although there is no congruence about the inhibition or activation of caspase-3 by propofol,\(^{10,26}\) our data emphasize the findings by Zhang et al.\(^{29}\) showing protective effects of propofol concerning caspase-3 activation.

To further unravel how diaphragm atrophy may develop in the current study, we examined protein synthesis through measurement of Akt-FoxO pathway, knowing that propofol has been reported to interact with pAkt/Akt ratio.\(^{30}\) We found a significant decrease in the ratio of phosphorylated to dephosphorylated Akt for both the SB animals and the animals under MV, indicating a reduction in protein synthesis under propofol sedation. There was, however, no concomitant decrease in phosphorylation of FoxO-1, which is downstream of Akt. Nevertheless, our data suggest that propofol may interact with the Akt pathway to induce atrophy in the diaphragm.

Clinical Implications

Propofol is a major agent for sedation in the ICU. Although the application is time limited due to the danger of the propofol infusion syndrome, leading to severe side effects as arrhythmia, hypotension, muscle necrosis, and acute renal failure with high mortality,\(^{31}\) it remains part of clinical practice for patients needing rapid awakening (e.g., after neurosurgery).\(^{31}\) Especially during the weaning process, it is used as a short-term sedative with a low-context sensitive half-life compared with benzodiazepines or many opioids. Besides improvement in lung function, the early activation of the diaphragm is meant to be the most effective way in protection against VIDD, with 5 min of SB per hour being sufficient to uphold protein synthesis.\(^{16,32}\) Although it may be possible that this contractile deficit is abundant after the end of propofol action, we also reported diaphragm muscle atrophy, suggesting that this contractile deficit is not transient and would not disappear upon cessation of propofol infusion. Hermans et al.\(^{11}\) have demonstrated that there is a correlation between the dosage of propofol and the amount of contractile dysfunction in ventilated patients. It should be considered that during ventilator weaning, which leads to gradual activation of the diaphragm, the usage of propofol might reduce diaphragm force instead of protecting it. Summarily, the described effects should be taken into account when longer-term propofol treatment of spontaneously breathing patients for periods of 24 h or even longer is considered, as the administration of propofol might counteract these effects and harm the active diaphragm.

Conclusion

Propofol administration in spontaneously breathing animals reduced contractile force and fiber size as if the animals had been ventilated. Notably, propofol does not demonstrate antioxidant properties that might prevent VIDD. Whether these data can be translated to humans has to be determined and warrants investigation.
Acknowledgments

Funding was provided by the medical faculty of the Rhenish Westphalian Technical University Aachen, Aachen, Germany (to Dr. Bruells), and by Fonds voor Wetenschappelijk Onderzoek-Flanders, Belgium (to Dr. Maes). The work was supported by AstraZeneca Pharmaceuticals, Leuven, Belgium.

Competing Interests

The authors declare no competing interests.

Correspondence

Address correspondence to Dr. Gayan-Ramirez: Katholieke Universiteit Leuven, Laboratory of Pneumology, O&N 1, Bus 706, Herestraat 49, 3000 Leuven, Belgium. ghislaine.gayan-ramirez@med.kuleuven.be. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY’s articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue. ANESTHESIOLOGY’s articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

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