Oxidative stress is required for mechanical ventilation-induced protease activation in the diaphragm

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Whidden MA, Smuder AJ, Wu M, Hudson MB, Nelson WB, Powers SK. Oxidative stress is required for mechanical ventilation-induced protease activation in the diaphragm. J Appl Physiol 108: 1376–1382, 2010. First published March 4, 2010; doi:10.1152/japplphysiol.00098.2010.—Prolonged mechanical ventilation (MV) results in diaphragmatic weakness due to fiber atrophy and contractile dysfunction. Recent work reveals that activation of the proteases calpain and caspase-3 is required for MV-induced diaphragmatic atrophy and contractile dysfunction. However, the mechanism(s) responsible for activation of these proteases remains unknown. To address this issue, we tested the hypothesis that oxidative stress is essential for the activation of calpain and caspase-3 in the diaphragm during MV. Cause-and-effect was established by prevention of MV-induced diaphragmatic oxidative stress using the antioxidant Trolox. Treatment of animals with Trolox prevented MV-induced protein oxidation and lipid peroxidation in the diaphragm. Importantly, the Trolox-mediated protection from MV-induced oxidative stress prevented the activation of calpain and caspase-3 in the diaphragm during MV. Furthermore, the avoidance of MV-induced oxidative stress not only averted the activation of these proteases but also rescued the diaphragm from MV-induced diaphragmatic myofiber atrophy and contractile dysfunction. Collectively, these findings support the prediction that oxidative stress is required for MV-induced activation of calpain and caspase-3 in the diaphragm and are consistent with the concept that antioxidant therapy can retard MV-induced diaphragmatic weakness.

skeletal muscle; antioxidant; calpain; caspase-3

MECHANICAL VENTILATION (MV) is used clinically to maintain blood gas homeostasis in patients who are incapable of maintaining adequate alveolar ventilation on their own (11). For patients with acute respiratory failure, MV is a life-saving process. However, while MV supports ventilation during periods of respiratory distress, removal from the ventilator (i.e., weaning) is often difficult. Although difficult weaning can be due to several factors, respiratory muscle weakness is predicted to play an important role (10, 13). Therefore, understanding the steps leading to MV-induced diaphragmatic weakness is important.

In reference to respiratory muscle weakness and MV, a causal link between MV and diaphragmatic weakness is clearly established, as prolonged MV promotes a rapid onset of diaphragmatic weakness due to diaphragmatic fiber atrophy and contractile dysfunction (16, 23, 30). This MV-induced diaphragmatic atrophy occurs as a result of an increase in proteolysis and a decrease in protein synthesis, with accelerated proteolysis playing a key role (17, 18, 29).

We previously showed that the ATP-dependent ubiquitin-proteasome pathway is involved in the degradation of myofibrillar protein during MV (2, 6, 20, 30). While the ubiquitin-proteasome pathway can degrade numerous cellular proteins, this proteolytic system does not degrade intact actomyosin complexes (9, 31). It follows that myofilaments must be released from the sarcomere to be degraded by the proteasome system (31, 35). In this regard, evidence indicates that calpain and caspase-3 are capable of producing actomyosin dissociation (7, 9, 31). Moreover, our work reveals that although several proteolytic systems are activated in the diaphragm during MV, the activation of calpain and/or caspase-3 is essential for MV-induced diaphragmatic atrophy (17, 18). Therefore, determining the mechanisms(s) responsible for MV-induced activation of calpain and caspase-3 in the diaphragm is an important step in developing a therapeutic approach to prevent the diaphragmatic weakness associated with prolonged MV.

Calpain is a calcium-dependent cysteine protease that is activated in skeletal muscle during conditions that promote skeletal muscle wasting (9). Indeed, calpains can degrade several cytoskeletal proteins that lead to the release of myofilaments (9). Although the control of calpain activation is complex, calpain activity is increased by factors that result in a sustained elevation of cytosolic calcium levels (9, 21).

Similar to calpain, caspase-3 is an endoprotease that degrades numerous muscle proteins and plays an important role in muscle protein degradation in a variety of wasting conditions (7). The control of caspase-3 activation is complicated and may involve several interconnected signaling pathways (21).

We and others previously showed that MV results in the rapid progression of diaphragmatic oxidative stress (2, 30, 37). In this regard, it is feasible that MV-induced oxidative stress can promote calpain and caspase-3 activation, because oxidants can increase cytosolic levels of calcium and trigger signaling pathways, leading to calpain and caspase-3 activation (22). Therefore, in the present study, we asked if oxidative stress is essential for MV-induced activation of calpain and caspase-3 in the diaphragm. On the basis of theory and preliminary experiments, we hypothesized that oxidative stress is required for activation of calpain and caspase-3 in the diaphragm during MV.

METHODS

Animals

Young adult (~5-mo-old) female Sprague-Dawley rats were maintained on a 12:12-h light-dark cycle, with food (AIN93 diet) and water available ad libitum throughout the experimental period. The Institutional Animal Care and Use Committee of the University of Florida approved these experiments.
Experimental Design

To test the hypothesis that oxidative stress is required for activation of calpain and caspase-3 in the diaphragm during MV, rats were randomly assigned to one of three experimental groups (n = 8/group): 1) acutely anesthetized control (CON), 2) 12 h mechanically ventilated (12MV), and 3) 12MV rats treated with Trolox (12MVT). The 12MV group received saline infusions during prolonged MV, whereas the 12MVT group was treated with the antioxidant Trolox before and during prolonged MV.

Experimental Protocol

Acutely anesthetized controls. Animals in the CON group were acutely anesthetized with pentobarbital sodium (60 mg/kg body wt ip). After the animal reached a surgical plane of anesthesia, the diaphragm was quickly removed and the costal diaphragm was divided into several experimental segments. A strip of the medial costal diaphragm was immediately used for in vitro contractile measurements, a separate section was frozen in isopentane cooled to the temperature of liquid nitrogen for histological measurements, and the remaining portions of the costal diaphragm were rapidly frozen in liquid nitrogen and stored at −80°C for subsequent biochemical analyses.

Mechanical ventilation. All surgical procedures were performed using aseptic techniques. Animals in the MV groups were anesthetized with pentobarbital sodium (60 mg/kg body wt ip), tracheostomized, and mechanically ventilated with a pressure-controlled ventilator (Servo Ventilator 300, Siemens) for 12 h with the following settings: upper airway pressure limit = 20 cmH2O, pressure control level above positive end-expiratory pressure = 4–6 cmH2O, respiratory rate = 80 breaths/min, and positive end-expiratory pressure = 1 cmH2O.

The carotid artery was cannulated to permit the continuous measurement of blood pressure and the collection of blood during the protocol. Arterial blood samples (100 μl per sample) were removed during hours 1, 3, 6, and 12 of MV and analyzed for arterial P2O (Pao2), PaCO2 (Paco2), and pH using an electronic blood-gas analyzer (GEM Premier 3000, Instrumentation Laboratory, Lexington, MA). Ventilator adjustments were made if Pao2 exceeded 40 Torr. Moreover, Paco2 was maintained at >60 Torr throughout the experiment by increasing the fraction of inspiratory O2 (22–26% O2).

A venous catheter was inserted into the jugular vein for continuous infusion of pentobarbital sodium (~10 mg·kg−1·h−1). Body temperature was maintained at 37°C by use of a recirculating heating blanket, and heart rate was monitored via a lead II electrocardiograph. Continuous care during the MV protocol included lubricating the eyes, expressing the bladder, removing airway mucus, rotating the animal, and passively moving the limbs. Glycopyrrolate (0.06 mg/kg im) was injected at 2-h intervals during MV to reduce airway secretions. Upon completion of MV, the diaphragm was quickly removed, and a strip of the medial costal diaphragm was used for in vitro contractile measurements, a section was stored in optimal cutting temperature compound for histology, and the remaining portion was frozen in liquid nitrogen and stored at −80°C for subsequent biochemical analyses.

Trolox administration. To prevent MV-induced diaphragmatic oxidative stress, we exogenously administered the water-soluble vitamin E analog Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid; Sigma Aldrich). Trolox is a potent antioxidant that rapidly crosses cell membranes and is a more effective antioxidant than its parent compound vitamin E (26). In a previous experiment, we performed dose-response studies and determined the appropriate Trolox treatment regimen for prevention of MV-induced oxidative stress in the diaphragm (2). Specifically, a loading dose of Trolox (20 mg/kg) was infused over a 5-min period, 20 min before the start of MV in the animals in the 12MVT group. During MV, a constant infusion of Trolox at 4 mg·kg−1·h−1 was maintained throughout the experiment.
Histological Measures

Myofiber CSA. Sections from frozen diaphragm samples were cut at 10 µm using a cryotome (Shandon, Pittsburgh, PA) and stained for dystrophin, myosin heavy chain (MHC) I, and MHC type IIa proteins for fiber CSA analysis, as described previously (18). CSA was determined using Scion software (NIH).

Statistical Analysis

Comparisons between groups for each dependent variable were made by a one-way ANOVA, and, when appropriate, Tukey’s honestly significant difference test was performed post hoc. Significance was established at \( P < 0.05 \). Data are presented as means ± SE.

RESULTS

Systemic and Biological Response to MV

There were no significant differences in body weight between the groups (0.305 ± 0.005, 0.294 ± 0.003, and 0.298 ± 0.004 kg for CON, 12MV, and 12MVT, respectively) before initiation of MV. Also, 12 h of MV did not alter body weight of the 12MV or 12MVT group. Heart rate (300–420 beats/min), systolic blood pressure (70–130 mmHg), and colonic (body) temperature (36–37°C) were maintained relatively constant during the 12 h of MV. Importantly, there were no significant differences between experimental groups in any of these measures at the completion of 12 h of MV (Table 1). \( \text{PaO}_2 \) and \( \text{PaCO}_2 \) were also maintained relatively constant during MV: \( \text{PaO}_2 \) ranged from 60–89 Torr, and \( \text{PaCO}_2 \) ranged from 34–46 Torr. Importantly, no significant differences existed between experimental groups in heart rate or arterial blood gases (Table 1).

At the completion of the MV protocols, there were no visual abnormalities of the lungs or peritoneal cavity, no evidence of lung infarction, and no evidence of infection, indicating that our aseptic surgical technique was successful.

Oxidative Stress

The efficacy of Trolox administration as a measure to prevent MV-induced diaphragmatic oxidative stress was determined by measuring protein oxidation and lipid peroxidation. Protein carbonyl formation is commonly used to measure oxidative stress and is a general indicator of protein oxidation. Twelve hours of MV resulted in a significant increase in diaphragmatic 4-HNE (\( P < 0.05 \)) compared with control (Fig. 1A). More importantly, Trolox administration significantly attenuated the increase in protein carbonyls in the diaphragm. Thus, Trolox administration completely attenuated MV-induced protein oxidation in the diaphragm.

4-HNE is the primary adduct formed during the lipid peroxidation cascade. Compared with control, 12 h of MV resulted in a significant increase in diaphragmatic 4-HNE (\( P < 0.05 \); Fig. 1B). Trolox administration significantly attenuated the accumulation of 4-HNE, as Trolox-treated animals had basal levels of diaphragmatic 4-HNE. Thus we have shown that Trolox administration during MV effectively inhibits diaphragmatic oxidative stress by the complete attenuation of protein oxidation and lipid peroxidation in the diaphragm of Trolox-treated MV animals.

Evidence of Protease Activation

Protease activation was analyzed via Western blotting of cleaved calpain-1 and cleaved caspase-3. As shown in Fig. 2, 12 h of MV resulted in a significant increase in active calpain-1 in the diaphragm (+66%, \( P < 0.05 \)), as detected by measuring the cleaved (active) 76-kDa calpain fragment. More importantly, Trolox completely prevented the MV-induced increase in cleaved calpain-1 in the diaphragm.

Table 1. HR, SBP, arterial blood gas tensions, and arterial pH after 12 h of MV

<table>
<thead>
<tr>
<th>Variable</th>
<th>12MV Mean ± SE</th>
<th>12MVT Mean ± SE</th>
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</thead>
<tbody>
<tr>
<td>HR, beats/min</td>
<td>339 ± 10</td>
<td>347 ± 7</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>101 ± 5</td>
<td>94 ± 5</td>
</tr>
<tr>
<td>Arterial ( \text{Po}_2 ), Torr</td>
<td>76 ± 4</td>
<td>79 ± 3</td>
</tr>
<tr>
<td>Arterial ( \text{Po}_2 ), Torr</td>
<td>43 ± 3</td>
<td>40 ± 1</td>
</tr>
<tr>
<td>Arterial pH</td>
<td>7.40 ± 0.01</td>
<td>7.42 ± 0.01</td>
</tr>
</tbody>
</table>

Values are means ± SE. HR, heart rate; SBP, systolic blood pressure; 12MV, 12 h of mechanical ventilation; 12MVT, 12MV + Trolox. Note no significant differences between the experimental groups in any of these physiological variables.

Fig. 1. Trolox administration prevents MV-induced oxidative damage to the diaphragm. A: levels of protein carbonyl formation in diaphragmatic myofibrillar proteins. Values are means ± SE. \(*P < 0.05\) vs. CON, \( \Psi P < 0.05 \) vs. 12MV. CON, control; 12MV, 12 h of mechanical ventilation; 12MVT, 12MV + Trolox. B: fold changes (vs. control) of 4-hydroxynonenal (4-HNE) accumulation in diaphragmatic proteins. Values are means ± SE. \(*P < 0.05\) vs. CON, \( \Psi P < 0.05 \) vs. 12MV.
The cleavage of procaspase-3 results in the activation of caspase-3. As shown in Fig. 3, 12 h of MV resulted in a significant increase in the 19-kDa cleaved (active) caspase-3 protein in the diaphragm ($P < 0.05$), and Trolox administration prevented the MV-induced activation of caspase-3 in the diaphragm.

Assay of Calpain and Caspase-3 Activation via Unique Degradation Products of $\alpha$-II Spectrin

$\alpha$-II spectrin is a cytoskeletal structural protein present in skeletal muscle. During periods of increased proteolytic activity, $\alpha$-II spectrin exhibits signature cleavage products that can be used to detect cleavage by calpain-1 and caspase-3. Specifically, the intact form of $\alpha$-II spectrin exists as a $\sim 250$-kDa protein and, upon degradation, yields 145-kDa (calpain-specific) and 120-kDa (caspase-3-specific) cleaved bands. Thus probing $\alpha$-II spectrin cleavage products can be used to reflect in vivo calpain-1 and caspase-3 activities. Assessment of $\alpha$-II spectrin cleavage for calpain-1 revealed increased cleavage following 12 h of MV ($P < 0.05$), and Trolox significantly attenuated the MV-induced increase in this calpain-specific cleavage product (Fig. 4A). In addition, 12 h of MV resulted in a significant increase in diaphragmatic levels of cleaved 120-kDa $\alpha$-II spectrin ($P < 0.05$; Fig. 4B). Trolox administration was effective in attenuating the MV-induced increase in the caspase-3 cleavage product of $\alpha$-II spectrin.

Diaphragmatic Contractile Dysfunction

Maximal isometric twitch force and force-frequency responses were measured in our experimental groups. Maximal isometric twitch force was significantly reduced by 30% following 12 h of MV ($6.73 \pm 0.35$ N/cm$^2$, $P < 0.05$) compared with controls ($9.55 \pm 0.28$ N/cm$^2$), and Trolox administration significantly attenuated the MV-induced decrease in diaphragmatic maximal isometric twitch force ($9.31 \pm 0.85$ N/cm$^2$).

The mean maximal force-frequency responses of in vitro costal diaphragm strips from CON, 12MV, and 12MVT animals are presented in Fig. 5. MV for 12 h shifted the force-frequency response downward compared with control. In fact, 12 h of MV resulted in a significant reduction ($P < 0.05$) in the specific force of the diaphragm compared with the CON group at all stimulation frequencies. Treatment with Trolox during MV significantly attenuated the MV-induced diaphragmatic...
contractile dysfunction at all stimulation frequencies. Therefore, Trolox administration is a potentially useful therapeutic approach to attenuate MV-induced diaphragmatic contractile dysfunction.

Diaphragmatic Myofiber Atrophy

Myofiber CSA was determined for individual fiber types in cross sections obtained from treatment diaphragms (Fig. 6A). MV for 12 h resulted in significant atrophy of type I, type IIa, and type IIb/IIx diaphragm myofibers (Fig. 6B). Importantly, Trolox administration attenuated atrophy in all diaphragm myofiber types during 12 h of MV. Therefore, prolonged MV resulted in a significant increase in diaphragmatic atrophy; however, Trolox was completely effective in preventing skeletal muscle loss during MV.

DISCUSSION

Overview of Principal Findings

It is established that prolonged MV results in a rapid onset of proteolysis, leading to diaphragmatic atrophy and weakness (8, 17, 23, 27, 30). Furthermore, it is also established that activation of calpain and caspase-3 plays an essential role in MV-induced diaphragmatic atrophy and contractile dysfunction (17, 18). However, the mechanism(s) responsible for the rapid activation of these proteases in the diaphragm during MV remains unknown. These experiments tested the hypothesis that oxidative stress is an upstream signal that is required to activate calpain and caspase-3 in the diaphragm during MV. Our results support this postulate, as administration of the antioxidant Trolox protected the diaphragm against oxidative stress and prevented the activation of calpain and caspase-3 in the diaphragm during MV. Importantly, this prevention of MV-induced activation of calpain and caspase-3 rescued the diaphragm from MV-induced diaphragmatic atrophy and contractile dysfunction. A detailed discussion of these new and important findings follows.

Clinical Significance of MV-Induced Diaphragmatic Weakness

All laboratory studies using a variety of animal models (i.e., baboons, rabbits, pigs, and rats) have consistently reported that...
prolonged MV results in diaphragmatic weakness due to atrophy and contractile dysfunction (1, 4, 14, 24, 27, 30). Indeed, the present investigation, along with previous reports, demonstrates that prolonged MV can induce diaphragmatic atrophy (e.g., ~20–30% reduction in myofiber CSA) within the first 12 h of MV (23). Importantly, recent work has also confirmed that MV also promotes rapid diaphragmatic atrophy in humans (16). Specifically, 18–69 h of MV in humans can produce significant diaphragmatic atrophy (e.g., ~50% reduction in myofiber CSA) in slow muscle fibers and fast diaphragm muscle fibers (16). Together, these results suggest that MV-induced diaphragmatic atrophy follows a similar time course in humans and animals.

The clinical significance of MV-induced diaphragmatic weakness is linked to the failure to liberate (i.e., wean) patients from the ventilator, as problems in weaning patients are common (3, 15). Failure to wean patients from MV is an important clinical problem, because prolonged time on the ventilator increases ventilator-associated complications and mortality (5, 15, 32–34). Although the potential causes of difficult weaning are numerous, it is believed that MV-induced diaphragmatic weakness is an important contributor (13, 36). Therefore, determining the signaling mechanisms responsible for MV-induced diaphragmatic weakness is important and forms the basis for the present study.

Oxidative Stress Is Required to Activate Calpain and Caspase-3 in the Diaphragm During Prolonged MV

We previously reported that the activation of calpain and caspase-3 plays an important role in MV-induced diaphragmatic atrophy (17, 18). Therefore, identifying the signal(s) responsible for MV-induced activation of calpain and caspase-3 is a required first step in developing a therapeutic target to prevent MV-induced diaphragmatic weakness. In this context, the present experiments provide the first evidence that oxidative stress is a required “upstream” signal to activate calpain and caspase-3 in the diaphragm during prolonged MV.

Intact, sarcomeric proteins cannot be degraded by the ubiquitin-proteasome system; therefore, myofilament release must occur prior to the breakdown of these proteins by the ubiquitin-proteasome system (19, 21, 22). Recent data indicate that calpain and caspase-3 are capable of mediating the disassembly of the sarcomere (7, 9, 31). Calpain is a cysteine protease that is activated in skeletal muscle during a variety of conditions that promote skeletal muscle wasting (e.g., prolonged disuse and sepsis) (9). Specifically, calpains are capable of degrading numerous muscle proteins that promote the release of myofilaments, and calpain activation may be a required step in all forms of muscle atrophy (9).

The regulation of calpain activity in cells is complex and involves several factors, including increased cytosolic calcium levels, phosphorylation, and the concentration of the endogenous calpain inhibitor calpastatin (9, 21). Because oxidative stress can disturb calcium homeostasis by increasing cellular levels of free calcium (12), we hypothesized that MV-induced oxidative stress would promote calpain activation in the diaphragm. Our results clearly support this prediction and reveal that oxidative stress is a requirement for calpain activation in the diaphragm during prolonged MV. However, our results do not expose whether this oxidative stress-induced activation of calpain in the diaphragm is due to increased cytosolic calcium levels alone or a combination of other factors that may contribute to calpain activation (e.g., phosphorylation). This unanswered question warrants additional research.

Similar to calpain, caspase-3 is a cysteine protease that can be activated in cells via a variety of signaling pathways. Importantly, caspase-3 activation has been shown to occur in skeletal muscles during several conditions that produce muscle wasting (7, 18). In this context, caspase-3 is capable of degrading actomyosin complexes in skeletal muscles (7), and caspase-3 activation is required for myonuclear apoptosis and myofiber atrophy in the diaphragm during prolonged MV (20).

Control of caspase-3 activity is multifaceted and involves several interconnected signaling pathways. In the case of MV-induced diaphragmatic atrophy, it is feasible that caspase-3 is activated by one or more upstream pathways, including the activation of caspase-8 (via signaling pathways from receptors on the cell membrane), caspase-12 (via calcium release from the endoplasmic reticulum), and/or activation of caspase-9 (via a mitochondrial pathway). In theory, oxidative stress could promote caspase-3 activation via one or all of these pathways, and future experiments are warranted to determine which of these signaling pathways trigger MV-induced caspase-3 activation in the diaphragm during prolonged MV.

Conclusions and Future Directions

Prolonged MV results in the rapid development of diaphragmatic weakness due to diaphragmatic atrophy and contractile dysfunction. In this regard, our prior work reveals that the activation of calpain and/or caspase-3 is a requirement for MV-induced diaphragmatic weakness (17, 18). The present experiments provide the first evidence that MV-induced oxidative stress is an essential upstream signal for calpain and caspase-3 activation in the diaphragm during prolonged MV. Moreover, our findings also demonstrate that antioxidant-mediated prevention of MV-induced oxidative stress rescues the diaphragm from MV-induced diaphragmatic atrophy and contractile dysfunction. Collectively, these results provide additional evidence that the prevention of oxidative stress is a potential therapeutic intervention to avert MV-induced diaphragmatic weakness.

The dominant cellular pathway(s) responsible for reactive oxygen species production in the diaphragm during prolonged MV remains unknown. This is unfortunate, because determining the source of reactive oxygen species production in the diaphragm during prolonged MV could be important in developing the optimal antioxidant strategy to prevent MV-induced oxidative stress. Clearly, this important issue warrants future experimental attention.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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