Trolox Attenuates Mechanical Ventilation–induced Diaphragmatic Dysfunction and Proteolysis

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Prolonged mechanical ventilation results in diaphragmatic oxidative injury, elevated proteolysis, fiber atrophy, and reduced force-generating capacity. We tested the hypothesis that antioxidant infusion during mechanical ventilation would function as an antioxidant to maintain redox balance within diaphragm muscle fibers and therefore prevent oxidative stress and subsequent proteolysis and contractile dysfunction. Sprague-Dawley rats were anesthetized, tracheostomized, and mechanically ventilated with 21% O_2 for 12 hours. The antioxidant Trolox was intravenously infused in a subset of ventilated animals. Compared with acutely anesthetized, nonventilated control animals, mechanical ventilation resulted in a significant reduction (–17%) in diaphragmatic maximal tetanic force. Importantly, Trolox completely attenuated this mechanical ventilation-induced diaphragmatic contractile deficit. Total diaphragmatic proteolysis was increased 105% in mechanical ventilation animals compared with controls. In contrast, diaphragmatic proteolysis did not differ between controls and mechanical ventilation–Trolox animals. Moreover, 20S proteasome activity in the diaphragm was elevated in the mechanical ventilation animals (+76%); Trolox treatment attenuated this mechanical ventilation-induced rise in protease activity. These results are consistent with the hypothesis that mechanical ventilation-induced oxidative stress is an important factor regulating mechanical ventilation-induced diaphragmatic proteolysis and contractile dysfunction. Our findings suggest that antioxidant therapy could be beneficial during prolonged mechanical ventilation.

Keywords: antioxidant; protein degradation; rat; weaning

Mechanical ventilation (MV) is used to sustain pulmonary gas exchange in patients who are incapable of maintaining adequate alveolar ventilation. The withdrawal of MV from patients is referred to as "weaning" and problems in weaning from MV are common (1). Numerous studies reveal that diaphragmatic weakness, due to both diaphragmatic atrophy and contractile dysfunction, is an important contributor to weaning difficulties (reviewed in References 2 and 3). Hence, developing strategies to oppose the deleterious effects of prolonged MV on the diaphragm is an important clinical goal.

The cellular mechanism(s) responsible for the rapid onset of MV-induced diaphragmatic atrophy and weakness remain(s) unclear. However, it is possible that MV-induced oxidative stress is an important contributor to both MV-induced proteolysis and contractile dysfunction. Indeed, our prior work has shown that MV is associated with a rapid onset of protein oxidation in diaphragm fibers (4, 5). This is significant because oxidative stress has been shown to promote disuse muscle atrophy (6, 7), and has been directly linked to activation of the ubiquitin–proteasome system of proteolysis (8–10). Moreover, oxidative stress reduces skeletal muscle force-generating capacity (reviewed in Reid [11]).

Therefore, the current study determined whether the prevention of MV-induced oxidative stress in the diaphragm would reduce MV-associated diaphragmatic proteolysis and contractile dysfunction. We hypothesized that infusion of an antioxidant would maintain redox balance within diaphragm muscle fibers during MV by functioning as a redox buffer and, thereby, prevent oxidative stress and subsequent proteolysis and contractile dysfunction.

Prevention of MV-induced oxidative stress in the diaphragm was achieved by infusing the antioxidant Trolox [(±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid; Trolox C Fluka/Sigma-Aldrich, St. Louis, MO]. Trolox is a water-soluble vitamin E analog that freely crosses cell membranes and has antioxidant properties (12–14). The effective antioxidant dose and infusion protocol for Trolox were determined in a series of preliminary experiments. Some of the results of these studies have been previously reported in the form of an abstract (15).

METHODS

Animals and Experimental Design

Female Sprague-Dawley rats (5.06 ± 0.12 months old, 284.5 ± 5.1 g) were randomly assigned to one of four groups: (*1*) Controls (CON, n = 8), (*2*) 12 hours of mechanical ventilation and saline infusion (MVS, n = 8), (*3*) 12 hours of MV and Trolox infusion (MVT, n = 8), and (*4*) 12 hours of anesthesia and spontaneous breathing with saline infusion (SBS, n = 8). All procedures were approved by the University of Florida (Gainesville, FL) Animal Care and Use Committee.

Control Animal Protocol

The control animals (CON) were free of intervention before receiving an acute intraperitoneal injection of sodium pentobarbital (65 mg/kg body weight) before diaphragm excision.

Mechanical Ventilation Protocol

Our MV protocol has previously been described (4, 16). Briefly, animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (65 mg/kg), tracheostomized and mechanically ventilated (control mode; tidal volume, about 0.55 ml/100 g; frequency of breathing, 80 breaths/minute; positive end-expiratory pressure, 1 cm H_2O) with a volume-cycled ventilator (Harvard Apparatus, Holliston, MA) for 12 hours. Body temperature was maintained at 37 ± 1°C.

Sodium pentobarbital (about 10 mg/kg per hour), and either Trolox or saline were infused intravenously during the treatment. Rats were continuously supervised and received constant care, including removal of airway mucus, passive limb movement, lubrication of the eyes, expression of the bladder, and enteral nutrition (Research Diets, New Brunswick, NJ). To reduce airway secretions, glycopyrrolate (0.04 mg/kg) was injected intramuscularly every 2 hours.

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In the MVT group, a priming dose of Trolox (20 mg/kg) was infused over a 5-minute period, 20 minutes before the start of MV. During MV, a constant infusion of Trolox at a rate of 4 mg/kg per hour (MVT) was maintained.

At the end of the 12-hour experimental period, the diaphragms were removed for immediate measurement of contractile muscle function and protein degradation as described below. Unused costal diaphragm tissue was frozen for biochemical analyses.

Spontaneous Breathing Protocol

Spontaneously breathing (SB) animals were anesthetized and received sham surgeries in the same manner as MV animals. These animals were tracheostomized and maintained on a surgical plane of anesthesia for 12 hours while continuing to breathe on their own.

Contractile Measurements

The optimal muscle length (L_o), force–frequency relationship, and fatigability of *in vitro* strips excised from the midcostal region of each diaphragm were measured as previously described (16, 17). Details of contractile muscle function measurements are available in the online supplement.

Protein Degradation

Concurrent with the contractile measurements, the total *in vitro* protein degradation in two separate diaphragm strips per animal was assessed by measuring the release of free tyrosine into the incubation medium, as previously described (4, 18). Tyrosine in the medium was assayed spectrofluorometrically (19).

20S Proteasome Activity

The *in vitro* chymotrypsin-like activity of the 20S proteasome was measured fluorometrically, using techniques described by Stein and coworkers (20).

Total Thiols and Glutathione

As an indicator of oxidative stress, we measured total thiol groups (21) and total glutathione (GSH; Cayman Chemical, Ann Arbor, MI) in the costal diaphragm.

Statistical Analysis

Group comparisons were made by one-way analysis of variance (Systat Software, Point Richmond, CA). Where significant differences were found, the Tukey HSD (honestly significantly different) test was implemented *post hoc*. Significance was established *a priori* at $p < 0.05$. Values given are means ± SEM.

RESULTS

Systemic and Biologic Responses to Treatment

The treatment protocol (MV or SB) did not significantly change body mass for any of the groups (Table 1), indicating that our schedule of nutrition and rehydration was adequate. The ratio of total costal diaphragm mass to final body mass was not significantly different between the four groups ($p = 0.5$).

There were no signs of infection in any animals, and only one MVS animal was eliminated from the study because of evidence of barotrauma to the lungs on postmortem examination. Systolic blood pressure was maintained at 70–110 mm Hg in all groups, and arterial pH, Po_2, and Pco_2 were maintained within physiological ranges for both MV groups as described in previous studies from our laboratory (4, 5, 16). The SB animals were mildly hypoxic, hypercapnic, and acidotic, an expected result postanesthesia. Body temperature was kept at 37 ± 1°C during the 12-hour protocol.

Effects of Anesthesia on Diaphragm Contractile Properties

The maximal tetanic force was not different between the SBS and CON groups (25.09 ± 0.41 versus 25.43 ± 0.50 N/cm^2, respectively; Table 2). Likewise, the force–frequency curves and fatigue data were similar between these two groups (Figures 1 and 2), and values for contractile parameters did not differ (Table 3). Thus, 12 hours of sodium pentobarbital anesthesia did not affect *in vitro* contractile properties of the diaphragm.

Effects of Mechanical Ventilation on Contractile Properties

Twelve hours of controlled MV reduced maximal tetanic force production by about 17% (21.01 ± 0.71 versus 25.43 ± 0.50 N/cm^2 in CON animals). The force–frequency curve of the MVS group was shifted downward and to the right of the CON group (Figure 1). This indicates a reduction in force generation at all stimulation frequencies tested. The fatigue protocol produced curves of similar shape for all groups (Figure 2), but the MVS group generated a significantly lower amount of force compared with CON, SBS, and MVT groups where indicated.

One-half relaxation time ($RT_{1/2}$) of maximal twitch was significantly shorter in the MVS group compared with CON, whereas one-half time to peak tension ($TPT_{1/2}$), rate of force development, and rate of relaxation were not different for either maximal twitch or maximal tetanic forces (Table 3).

Effects of Trolox on Contractile Properties

Trolox supplementation during 12 hours of MV completely attenuated the loss of maximal force generation. The MVT group was not significantly different from the CON group at any stimulation frequency tested (Figure 1). Animals receiving Trolox during MV maintained a greater force-generating ability after the fatigue protocol compared with the unsupplemented MVS group (Figure 2). Trolox during MV significantly prolonged the rate of relaxation of maximal twitch compared with the CON group, but did not affect other contractile parameters (Table 3).

Protein Degradation

Twelve hours of controlled MV significantly elevated total *in vitro* protein degradation (+105%), as measured by the release of free tyrosine, compared with the CON group (Figure 3). However, protein degradation of the MVT group was not significantly different from the CON group ($p = 0.797$). There were no

TABLE 1. BODY WEIGHTS OF CONTROL, SPONTANEOUSLY BREATHING, AND MECHANICAL VENTILATION ANIMALS

	CON	SBS	MVS	MVT
Initial body mass, g	264.38 ± 5.16	276.88 ± 5.29	282.25 ± 3.33	300.13 ± 6.37*,†
Final body mass, g	264.38 ± 5.16	280.56 ± 5.52	286.38 ± 3.18*	306.75 ± 6.42*,†

Definition of abbreviations: CON = control animals; SBS = spontaneously breathing animals; MVS = mechanical ventilation animals; MVT = mechanical ventilation animals receiving Trolox.

Values represent means ± SEM.

* Significantly different from CON group, $p < 0.05$.

† Significantly different from SBS group, $p < 0.05$.

TABLE 2. DIAPHRAGMATIC MAXIMAL ISOMETRIC TWITCH AND TETANIC FORCE OF CONTROL, SPONTANEOUSLY BREATHING, AND MECHANICAL VENTILATION ANIMALS

	CON	SBS	MVS	MVT
Maximal isometric twitch force, N/cm^2	7.24 ± 0.14	6.79 ± 0.30	5.65 ± 0.28 *,†,‡	7.04 ± 0.25
Maximal isometric tetanic force, N/cm^2	25.43 ± 0.50	25.09 ± 0.41	21.01 ± 0.71*,†,‡	25.49 ± 0.50

For definition of abbreviations *see* Table 1.
Values represent means ± SEM.
* Significantly different from CON group, $p < 0.05$.
† Significantly different from SBS group, $p < 0.05$.
‡ Significantly different from MVT group, $p < 0.05$.

significant differences in protein degradation between the CON and SBS groups ($p = 0.351$).

20S Proteasome Activity

The chymotrypsin-like activity of the 20S proteasome was significantly increased in the MVS group compared with the CON group (+76%) (Figure 4). Trolox attenuated the MV-induced increase in proteasome activity (+26% compared with CON, $p = 0.647$).

Oxidative Stress

Total thiols in the diaphragm were significantly reduced with 12 hours of MV, indicative of oxidative stress (Table 4). However, total thiols were not significantly different between the MVS and MVT groups, suggesting that Trolox did not prevent the MV-induced reduction in total thiol groups in the diaphragm. Similarly, total GSH was significantly reduced after MV treatment, but total GSH levels in the diaphragm did not differ between MVS and MVT treatments (Table 4).

DISCUSSION

Major Findings

This is the first investigation to reveal that treatment with the antioxidant Trolox can retard the deleterious effects of prolonged MV on the diaphragm. Indeed, our results divulge that therapeutic delivery of Trolox during controlled MV attenuates both contractile dysfunction and muscle proteolysis in the diaphragm. Moreover, the elevation in MV-induced diaphragmatic proteolysis was due, in part, to increased chymotrypsin-like activity of the 20S proteasome. Importantly, administration of Trolox during MV prevented the MV-induced increase in 20S proteasome activity. A brief discussion of these findings follows.

MV-induced Oxidative Stress in the Diaphragm

It is well established that unloaded locomotor skeletal muscle is susceptible to oxidative stress during periods of disuse (6, 7, 22). Similarly, during MV, the diaphragm muscle is unloaded or inactive, passively shortened, and oxidative injury occurs within 6 hours of the onset of MV (4, 5). To date, our current or previous experiments have not identified the oxidant pathways in the diaphragm responsible for MV-induced oxidative stress. However, work by Lawler and colleagues (22) reveals that oxidative stress accompanies the unloading of locomotor skeletal muscle via a decrease in muscle antioxidant capacity and an increase in superoxide and hydrogen peroxide generation. In theory, numerous oxidant-producing pathways exist in skeletal muscle including mitochondrial, NADPH oxidase, and xanthine oxidase pathways. Nonetheless, the specific oxidant-producing pathway responsible for disuse-induced redox disturbance in hindlimb muscles was not identified in the work of Lawler and coworkers (22). Therefore, although it is clear that prolonged MV results in oxidative stress in the diaphragm, the pathways responsible for this oxidant production are unclear and remain an important area for future research.

In the current experiments, we measured diaphragmatic levels of total thiols and total GSH as markers of oxidative damage in the diaphragm. Our results indicated that MV resulted in

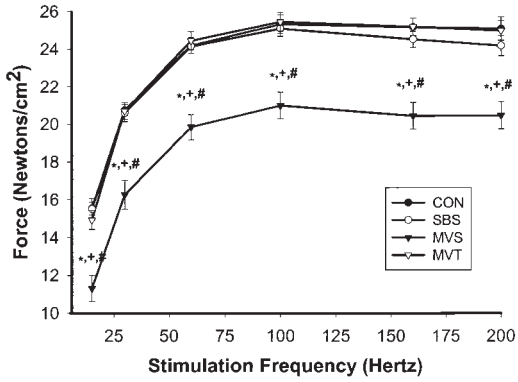

Figure 1. Force–frequency curves of *in vitro* diaphragm strips from control (CON), spontaneously breathing (SBS), mechanical ventilation (MVS), and mechanical ventilation animals receiving Trolox (MVT). Values represent means ± SEM. *Significantly different from CON group, $p < 0.05$; +significantly different from SBS group, $p < 0.05$; #significantly different from MVT group, $p < 0.05$.

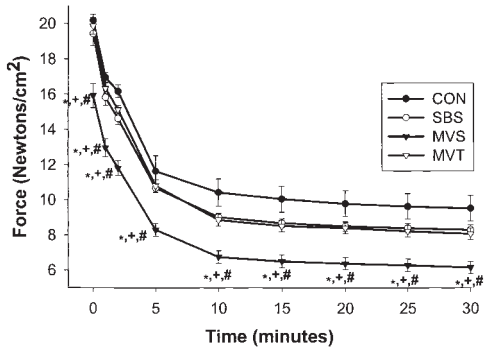

Figure 2. Comparison of fatigue development of *in vitro* diaphragm strips of control (CON), spontaneously breathing (SBS), mechanical ventilation (MVS), and mechanical ventilation animals receiving Trolox (MVT) during a 30-minute fatigue protocol. Values represent means ± SEM. *Significantly different from CON group, $p < 0.05$; +significantly different from SBS group, $p < 0.05$; #significantly different from MVT group, $p < 0.05$.

TABLE 3. DIAPHRAGMATIC CONTRACTILE PARAMETERS OF MAXIMAL ISOMETRIC TWITCH AND TETANIC FORCES OF CONTROL, SPONTANEOUSLY BREATHING, AND MECHANICAL VENTILATION ANIMALS

	CON	SBS	MVS	MVT
		Twitch		
$TPT_{1/2}$	0.018 ± 0.000	0.018 ± 0.001	0.017 ± 0.000	0.018 ± 0.000
$RT_{1/2}$	0.044 ± 0.001	0.040 ± 0.001	0.034 ± 0.003*	0.04 ± 0.001
+dp/dt	332.29 ± 27.368	420.97 ± 31.176	415.85 ± 57.784	459.52 ± 18.567
−dp/dt	−149.26 ± 9.247	−177.84 ± 9.696	−207.93 ± 28.890	−204.21 ± 7.845*
		Tetanic		
$TPT_{1/2}$	0.064 ± 0.002	0.062 ± 0.001	0.056 ± 0.003	0.059 ± 0.002
$RT_{1/2}$	0.064 ± 0.002	0.062 ± 0.003	0.062 ± 0.003	0.065 ± 0.002
+dp/dt	423.71 ± 34.738	527.67 ± 41.175	515.38 ± 95.654	570.18 ± 20.668
−dp/dt	−544.55 ± 50.860	−723.04 ± 36.225	−703.53 ± 157.30	−740.20 ± 12.091

Definition of abbreviations: +dp/dt = rate of force development; −dp/dt = rate of force relaxation; CON = control animals; MVS = mechanical ventilation animals; MVT = mechanical ventilation animals receiving Trolox; RT = relaxation time (milliseconds); SBS = spontaneously breathing animals; TPT = time to peak tension (milliseconds).

Values represent means ± SEM.

* Significantly different from CON group, $p < 0.05$.

a significant decrease in both markers in the diaphragm; this observation is consistent with an increase in oxidative stress in the diaphragm (Table 4). However, to our surprise, Trolox did not prevent the reduction in both total thiol groups or total GSH in the diaphragm during MV. In this regard, it is possible that Trolox infusion prevented MV-induced oxidative modification of proteins in ways that were not reflected in our measurements of total GSH or thiol groups. Oxidative injury can alter cellular homeostasis by damaging all types of biomolecules (i.e., DNA, lipids, and proteins) (23). It follows that detection of oxidative modification of biomolecules is dependent on the assays to measure such damage. For example, measurement of total thiols in cells would not detect protein damage such as the formation of protein carbonyls or protein cross-linking. Moreover, measurement of total thiols or GSH in the tissue does not indicate whether lipid peroxidation occurred in the diaphragm during prolonged MV. It is possible that Trolox provided diaphragmatic protection against MV-induced oxidative stress, but such protection was not detectable by the measurement of total thiols or GSH alone. Tissue limitations prevented measurement of such oxidative injury markers. Nonetheless, we postulate that Trolox functioned in our experiments as an antioxidant to prevent MV-induced oxidative injury in the diaphragm. In support of this notion, numerous studies indicate that Trolox reduces oxidative stress in multiple cell types induced by cumene hydroperoxide (24), methylmercury intoxication (25), and other oxyradicals (12–14, 26). Moreover, Trolox has been shown to reduce oxidative damage in cardiac muscle during ischemia–reperfusion insults (14, 27, 28). Collectively, these data support the role of Trolox as a biologically active antioxidant.

MV and Diaphragmatic Contractile Dysfunction

Our diaphragmatic contractile results (in saline-infused animals) agree with previous reports indicating that prolonged MV promotes diaphragmatic contractile dysfunction in rats (16, 29–31), baboons (32), piglets (33), and rabbits (34, 35). In the present study, maximal tetanic specific tension was decreased about 17% with 12 hours of controlled MV. A new and important finding in the current study is that Trolox infusion during MV completely attenuated the decrease in both submaximal and maximal spe-

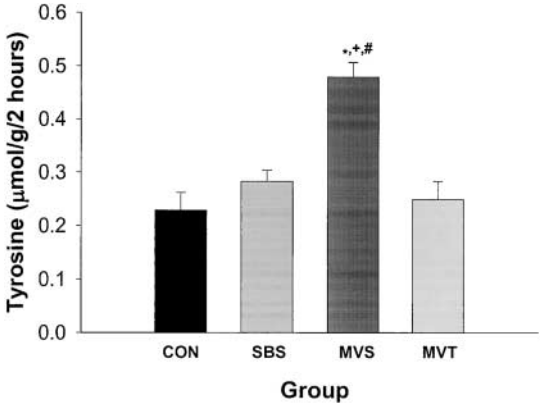

Figure 3. Effect of mechanical ventilation and Trolox supplementation on total *in vitro* protein degradation as measured by the rate of tyrosine release from diaphragm muscle strips per wet weight of muscle in 2 hours. CON = control; SBS = spontaneously breathing; MVS = mechanical ventilation; MVT = mechanical ventilation animals receiving Trolox. Values represent means ± SEM. *Significantly different from CON group, $p < 0.05$; +significantly different from SBS group, $p < 0.05$; #significantly different from MVT group, $p < 0.05$.

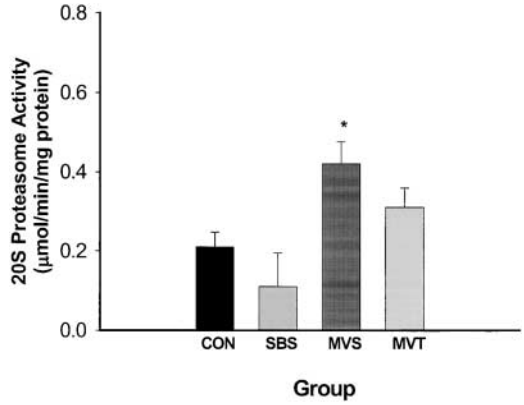

Figure 4. Effect of mechanical ventilation and Trolox supplementation on chymotrypsin-like activity of the 20S proteasome in the diaphragm of control (CON), spontaneously breathing (SBS), mechanical ventilation (MVS), and mechanical ventilation animals receiving Trolox (MVT). Values represent means ± SEM. *Significantly different from CON group, $p < 0.05$.

TABLE 4. TOTAL THIOLS AND TOTAL GLUTATHIONE CONCENTRATIONS OF CONTROL, SPONTANEOUSLY BREATHING, AND MECHANICAL VENTILATION ANIMALS

	CON	SBS	MVS	MVT
Total thiols, nmol/mg protein	157.34 ± 6.45	144.06 ± 6.89	134.83 ± 6.90*	122.0 ± 6.45*
Total GSH, mmol/g	1.15 ± 0.12	0.68 ± 0.05*	0.47 ± 0.08*	0.40 ± 0.05*

Definition of abbreviations: CON = control animals; GSH = glutathione; MVS = mechanical ventilation animals; MVT = mechanical ventilation animals receiving Trolox; SBS = spontaneously breathing animals.

Values represent means ± SEM.

* Significantly different from CON group, $p < 0.05$.

cific tension that normally occurs after prolonged MV. Specifically, diaphragmatic contractile function in the Trolox-treated MV animals did not differ from diaphragmatic function in CON or SBS animals with respect to maximal tetanic specific tension, maximal twitch specific tension, submaximal specific force production (i.e., 15–60 Hz), and diaphragm fatigue properties. The specific mechanism(s) responsible for the Trolox-mediated diaphragmatic protection is unclear but several possibilities exist, as detailed below.

First, oxidative stress-induced changes in calcium regulation are a potential contributor to oxidant-mediated contractile dysfunction (reviewed in Reid [11]). In this regard, it is well established that several sarcoplasmic reticulum proteins are sensitive to redox modulation, including the ryanodine-sensitive calcium channel (36) and the sarcoplasmic/endoplasmic reticulum calcium-dependent ATPase. Indeed, a number of redox sensitive sulfhydryls exist near the sarcoplasmic/endoplasmic reticulum calcium-dependent ATPase active site and are known to regulate calcium pump activity (reviewed in Reid [11]). Generally, oxidative stress in skeletal muscles leads to an increase in cytosolic calcium levels and a slowed rate of calcium uptake into the sarcoplasmic reticulum (11). Paradoxically, we found that the $RT_{1/2}$ for twitch contractions was significantly reduced in the MVS group, suggesting a greater rate of calcium uptake after MV. The significance of this finding is unclear because no other contractile characteristics related to calcium kinetics differed between control and MVS.

Another important target for oxidative damage in muscle is myofilaments (37). Several studies reveal that myofilament function can be significantly altered by exposure to reactive oxygen and nitrogen species (38–40). Several important contractile and regulatory proteins could participate in these responses. For example, the S1 portion of the myosin heavy chain molecule contains several redox-sensitive sites and oxidation could reduce the number of myosin cross-bridges in the strong binding state, resulting in reduced force production (reviewed in Reid [11]). Troponin also exhibits redox sensitivity whereby oxidation could influence calcium sensitivity (11). Other myofilaments may also exhibit redox sensitivity (e.g., myosin light chains, actin, and tropomyosin) but functional details of this redox influence remain poorly defined.

Finally, it is also possible that MV-induced oxidative stress promoted proteolysis in the diaphragm, resulting in degradation of important muscle proteins involved in force production. For example, degradation of key cytoskeletal or contractile proteins could reduce force-generating capacity in the muscle. Our results indicate that Trolox administration reduced the rate of total muscle proteolysis in the diaphragm during prolonged MV. Nonetheless, whether protection against protein breakdown played a critical role in the preservation of diaphragmatic force production is unclear.

In summary, the contractile dysfunction produced by oxidative stress in skeletal muscle may be mediated by oxidant action on more than one molecular target. Indeed, the fact that numerous skeletal muscle proteins are redox sensitive argues against a single site of redox action in muscle. Hence, it is possible that Trolox-mediated protection against MV-induced diaphragmatic contractile dysfunction was achieved by the prevention of oxidative injury at several molecular sites within the muscle.

MV and Proteolysis

Twelve hours of controlled MV significantly increased (+105%) the release of tyrosine from *in vitro* diaphragm strips. This agrees with a previous study from our laboratory reporting increased tyrosine release after 18 hours of MV (4). Because tyrosine is neither synthesized nor degraded by skeletal muscle, tyrosine release can be used as an indicator of total protein degradation (41). In our previous investigation, we determined that 20S proteasome activity was elevated, indicating a contribution of this pathway to diaphragmatic proteolysis during MV (4).

Importantly, in the current experiments, Trolox attenuated the increase in total protein degradation induced by MV (Figure 3). Likewise, the chymotrypsin-like activity of the 20S proteasome was elevated during MV, but this increase was prevented with Trolox (Figure 4). Many studies indicate that the proteasome is responsible for about 70–80% of the increased cellular protein degradation after an oxidative stress (8, 9, 42). More specifically, it appears the 20S proteasome is responsible for the degradation of oxidized proteins because the 26S proteasome can be inhibited by oxidative stress (43). Recognition of exposed hydrophobic patches is the proposed mechanism by which the proteasome selectively degrades oxidatively modified proteins (44). Oxidative damage to a protein leads to partial unfolding and exposure of normally shielded internal hydrophobic patches that are recognized by the proteasome, which catalyzes the degradation of that protein.

The diaphragmatic atrophy and contractile dysfunction that occur with prolonged MV are likely the result of increased oxidative modification of proteins, leading to proteolytic attack and degradation. This loss of protein, especially contractile protein, would result in atrophy and decreases in maximal force production. Clinically, this would manifest as difficulty in weaning from the mechanical ventilator.

Conclusions

Our results support earlier conclusions that short-term controlled MV leads to diaphragmatic contractile dysfunction and increased protein degradation. Importantly, our novel results clearly demonstrate that an antioxidant, Trolox, effectively prevents MV-induced contractile impairments and proteolysis in the diaphragm during MV. Oxidative damage and atrophy are implicated in MV-induced contractile deficits. Oxidative damage to proteins during MV likely increases proteolytic degradation, which would contribute to diaphragmatic weakness. Trolox effectively spares the unloaded diaphragm from contractile dysfunction and protein degradation during 12 hours of controlled MV. The use of an antioxidant such as Trolox may prove beneficial in the clinical setting, where weaning difficulties are encoun-

tered due to diaphragmatic atrophy and weakness. This is an important clinical issue that warrants further investigation.

***Conflict of Interest Statement*:** J.L.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; D.S.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; R.A.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; D.V.G. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; D.F. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; K.C.D. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; M.D. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; T.Y. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; S.K.P. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

References

1. Esteban A, Frutos F, Tobin MJ, Alia I, Solsona JF, Valverdu I, Fernandez R, de la Cal MA, Benito S, Tomas R, Spanish Lung Failure Collaborative Group. A comparison of four methods of weaning patients from mechanical ventilation. *N Engl J Med* 1995;332:345–350.
2. Gayan-Ramirez G, Decramer M. Effects of mechanical ventilation on diaphragm function and biology. *Eur Respir J* 2002;20:1579–1586.
3. Vassilakopoulos T, Petrof BJ. Ventilator-induced diaphragmatic dysfunction. *Am J Respir Crit Care Med* 2004;169:336–341.
4. Shanely RA, Zergeroglu MA, Lennon SL, Sugiura T, Yimlamai T, Enns D, Belcastro A, Powers SK. Mechanical ventilation-induced diaphragmatic atrophy is associated with oxidative injury and increased proteolytic activity. *Am J Respir Crit Care Med* 2002;166:1369–1374.
5. Zergeroglu MA, McKenzie MJ, Shanely RA, Van Gammeren D, DeRuisseau KC, Powers SK. Mechanical ventilation-induced oxidative stress in the diaphragm. *J Appl Physiol* 2003;95:1116–1124.
6. Kondo H, Kodama J, Kishibe T, Itokawa Y. Oxidative stress during recovery from muscle atrophy. *FEBS Lett* 1993a;326:189–191.
7. Kondo H, Nakagaki I, Sasaki S, Hori S, Itokawa Y. Mechanism of oxidative stress in skeletal muscle atrophied by immobilization. *Am J Physiol* 1993b;265:E839–E844.
8. Grune T, Reinheckel T, Joshi M, Davies KJA. Proteolysis in cultured liver epithelial cells during oxidative stress: role of the multicatalytic proteinase complex, proteasome. *J Biol Chem* 1995;270:2344–2351.
9. Grune T, Reinheckel T, Davies KJA. Degradation of oxidized proteins in K562 human hematopoietic cells by proteasome. *J Biol Chem* 1996; 271:15504–15509.
10. Li YP, Chen Y, Li AS, Reid MB. Hydrogen peroxide stimulates ubiquitin-conjugating activity and expression of genes for specific E2 and E3 proteins in skeletal muscle myotubes. *Am J Physiol Cell Physiol* 2003;285:C806–C812.
11. Reid MB. Redox modulation of skeletal muscle contraction: what we know and what we don't. *J Appl Physiol* 2001;90:724–731.
12. Wu TW, Hashimoto N, Wu J, Carey D, Li RK, Mickle DA, Weisel RD. The cytoprotective effect of Trolox demonstrated with three types of human cells. *Biochem Cell Biol* 1990;68:1189–1194.
13. Zeng L-H, Wu J, Carey D, Wu T-W. Trolox and ascorbate: are they synergistic in protecting liver cells *in vitro* and *in vivo*? *Biochem Cell Biol* 1991;69:198–201.
14. Walker MK, Vergely C, Lecour S, Abadie C, Maupoil V, Rochette L. Vitamin E analogues reduce the incidence of ventricular fibrillations and scavenge free radicals. *Fundam Clin Pharmacol* 1998;12:164–172.
15. Van Gammeren D, Betters JL, Shanely RA, Falk DJ, DeRuisseau KC, Powers SK, Criswell DC. Trolox attenuates mechanical ventilation-induced contractile dysfunction and proteasome activity of the diaphragm: experimental biology meeting [abstract]. *FASEB J* 2004;18: A746.
16. Powers SK, Shanely RA, Coombs JS, Koesterer TJ, McKenzie M, Van Gammeren D, Cicale M, Dodd SL. Mechanical ventilation results in progressive contractile dysfunction in the diaphragm. *J Appl Physiol* 2002;92:1851–1858.
17. Criswell DS, Shanely RA, Betters JJ, McKenzie MJ, Sellman JE, Van Gammeren DL, Powers SK. Cumulative effects of aging and mechanical ventilation on *in vitro* diaphragm function. *Chest* 2003;124:2302–2308.
18. Tischler ME, Desautels M, Goldberg AL. Does leucine, leucyl-tRNA, or some metabolite of leucine regulate protein synthesis and degradation in skeletal and cardiac muscle? *J Biol Chem* 1982;257:1613–1621.
19. Waalkes TP, Udenfriend S. A fluorometric method for the estimation of tyrosine in plasma and tissues. *J Lab Clin Med* 1957;50:733–736.
20. Stein RL, Melandri F, Dick L. Kinetic characterization of the chymotryptic activity of the 20S proteasome. *Biochemistry* 1996;35:3899–3908.
21. Vincent HK, Powers SK, Stewart DJ, Demirel HA, Shanely RA, Naito H. Short-term exercise training improves diaphragm antioxidant capacity and endurance. *Eur J Appl Physiol* 2000;81:67–74.
22. Lawler JM, Song W, Demaree SR. Hindlimb unloading increases oxidative stress and disrupts antioxidant capacity in skeletal muscle. *Free Radic Biol Med* 2003;35:9–16.
23. Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. London: Oxford University Press; 1999.
24. Persoon-Rothert M, Egas-Kenniphaas JM, van der Valk-Kokshoorn EJ, Mauve I, van der Laarse A. Prevention of cumene hydroperoxide induced oxidative stress in cultured neonatal rat myocytes by scavengers and enzyme inhibitors. *J Mol Cell Cardiol* 1990;22:1147–1155.
25. Usuki F, Yasutake A, Umehara F, Tokunaga H, Matsumoto M, Eto K, Ishiura S, Higuchi I. *In vivo* protection of a water-soluble derivative of vitamin E, Trolox, against methylmercury-intoxication in the rat. *Neurosci Lett* 2001;304:199–203.
26. Wu TW, Hashimoto N, Au JX, Wu J, Mickle DA, Carey D. Trolox protects rat hepatocytes against oxyradical damage and the ischemic rat liver from reperfusion injury. *Hepatology* 1991;13:575–580.
27. Klein HH, Pich S, Schuff-Werner P, Niedmann P, Blattmann U, Nebendahl K. The effects of Trolox, a water-soluble vitamin E analogue, in regionally ischemic, reperfused porcine hearts. *Int J Cardiol* 1991;32:291–301.
28. Sagach VF, Scrosati M, Fielding J, Rossoni G, Galli C, Visioli F. The water-soluble vitamin E analog Trolox protects against ischemia/reperfusion damage *in vitro* and *ex vivo*: a comparison with vitamin E. *Pharmacol Res* 2002;45:435–439.
29. Le Bourdelles G, Viires N, Boczkowski J, Seta N, Pavlovic D, Aubier M. Effects of mechanical ventilation on diaphragmatic contractile properties in rats. *Am J Respir Crit Care Med* 1994;149:1539–1544.
30. Shanely RA, Coombes JS, Zergeroglu M, Webb AI, Powers SK. Short-duration mechanical ventilation enhances diaphragmatic fatigue resistance but impairs force production. *Chest* 2003;123:195–201.
31. Gayan-Ramirez G, de Paepe K, Cadot P, Decramer M. Detrimental effects of short-term mechanical ventilation on diaphragm function and IGF-1 mRNA in rats. *Intensive Care Med* 2003;29:825–833.
32. Anzueto A, Peters JI, Tobin MJ, De Los Santos R, Seidenfeld JJ, Moore G, Cox WJ, Coalson JJ. Effects of prolonged controlled mechanical ventilation on diaphragmatic function in healthy adult baboons. *Crit Care Med* 1997;25:1187–1190.
33. Radell PJ, Remahl S, Nichols DG, Eriksson LI. Effects of prolonged mechanical ventilation and inactivity on piglet diaphragm function. *Intensive Care Med* 2002;28:358–364.
34. Sassoon CSH, Caiozzo VJ, Manka A, Sieck GC. Altered diaphragm contractile properties with controlled mechanical ventilation. *J Appl Physiol* 2002;92:2585–2595.
35. Capdevila X, Lopez S, Bernard N, Rabischong E, Ramonatxo M, Martinazzo G, Prefaut C. Effects of controlled mechanical ventilation on respiratory muscle contractile properties in rabbits. *Intensive Care Med* 2003;29:103–110.
36. Hamilton SL, Reid MB. RyR1 modulation by oxidation and calmodulin. *Antioxid Redox Signal* 2000;2:41–45.
37. Haycock JW, Jones P, Harris JB, Mantle D. Differential susceptibility of human skeletal muscle proteins to free radical induced oxidative damage: a histochemical, immunocytochemical, and electron microscopical study *in vitro*. *Acta Neuropathol (Berl)* 1996;92:331–340.
38. Perkins WJ, Han YS, Sieck GC. Skeletal muscle force and actomyosin ATPase activity reduced by nitric oxide donor. *J Appl Physiol* 1997; 83:1326–1332.
39. Andrade FH, Reid MB, Allen DG, Westerblad H. Effect of hydrogen peroxide and dithiothreitol on contractile function of single skeletal muscle fibres from the mouse. *J Physiol* 1998;509:565–575.
40. Andrade FH, Reid MB, Allen DG, Westerblad H. Effect of nitric oxide on single skeletal muscle fibres from the mouse. *J Physiol* 1998;509: 577–586.
41. Lowell BB, Ruderman NB, Goodman MN. Regulation of myofibrillar protein degradation in rat skeletal muscle during brief and prolonged starvation. *Metabolism* 1986;35:1121–1127.
42. Grune T, Davies KJA. Breakdown of oxidized proteins as a part of secondary antioxidant defenses in mammalian cells. *Biofactors* 1997; 6:165–172.
43. Reinheckel T, Sitte N, Ulrich O, Kuckelkorn U, Grune T, Davies KJA. Comparative resistance of the 20S and 26S proteasome to oxidative stress. *Biochem J* 1998;335:637–642.
44. Grune T, Reinheckel T, Davies KJA. Degradation of oxidized proteins in mammalian cells. *FASEB J* 1997;11:526–534.