Cervical spinal cord injury exacerberates ventilator-induced diaphragm dysfunction

Ashley J. Smuder,1 Elisa J. Gonzalez-Rothi,2 Oh Sung Kwon,1 Aaron B. Morton,1 Kurt J. Sollanek,1 Scott K. Powers,1 and David D. Fuller2

1Department of Applied Physiology and Kinesiology, University of Florida, Gainesville, Florida; and 2Department of Physical Therapy, University of Florida, Gainesville, Florida

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Cervical spinal cord injury (SCI) affects 11,000 new individuals every year with approximately half occurring in the cervical region (2, 6, 47). Injuries to the cervical spinal cord almost always compromise ventilation resulting in increased morbidity and mortality (4, 20, 31). Respiratory impairments are triggered initially by disruption of neuronal input to the diaphragm, and diaphragm atrophy and contractile dysfunction can occur rapidly (14, 26). Mechanical ventilation (MV) is often required to maintain pulmonary gas exchange after SCI, particularly with injury to the high cervical spinal cord (5, 12, 57). Although MV is a life-saving intervention, it can result in rapid development of diaphragm atrophy and impaired contractility, a condition known as ventilator-induced diaphragm dysfunction (VIDD) (25, 35, 45). VIDD occurs in animals and humans, and results from increased diaphragm reactive oxygen species (ROS) production and accelerated protein degradation (3, 24, 25, 44, 56).

Reports indicate that the duration of time a patient requires ventilatory support is directly related to the level of spinal cord injury, with patients injured at C1–C4 remaining ventilator dependent the longest (1). In addition, patients with cervical SCI also have a higher incidence (71%) of weaning failure compared with patients with thoracic (19%) and lumbar (10%) injuries (5, 12, 57). While neurologic impairments trigger the need for MV support after SCI, there is often a modest degree of phrenic motor recovery after incomplete cervical SCI (22). However, translating increases in phrenic neuromotor output to meaningful “respiratory recovery” and/or ventilator weaning is likely to be complicated by the onset of respiratory muscle dysfunction. VIDD is predicted to be a primary contributor to the inability to wean patients from the ventilator (13). To date, studies of VIDD have focused on alterations to diaphragm muscle structure and function in spinal-intact humans and rodent models, and there is no information currently available regarding the combined impact of SCI and MV on diaphragm function. Accordingly, we used an animal model of cervical SCI to simulate the condition in which ventilator support is provided following an acute SCI. Rats received a high cervical (C2) spinal hemileision, which results in transient paralyses of the ipsilateral hemidiaphragm (41) along with rapid proteolysis (14). We hypothesized that cervical SCI occurring prior to a sustained period of MV would exacerbate VIDD as a result of amplified oxidative stress. The prominent contribution of ROS production to VIDD (3, 24, 25, 44, 56) led us to further hypothesize that antioxidant administration could mitigate the impact of MV on the diaphragm following SCI.

METHODS

Animals and Experimental Design

To investigate the effects of MV and SCI on diaphragm function we performed two separate experiments. Experiment 1 was performed to determine if SCI exacerbates VIDD, and the goal of experiment 2 was to determine if antioxidant treatment protects the diaphragm against the effects of SCI alone or SCI + MV. Prior studies have established that diaphragm dysfunction following SCI or MV develops rapidly in both male and female rats (14, 48); we elected to use young adult (~6 mo old) female Sprague-Dawley rats for these experiments. The Institutional Animal Care and Use Committee of the University of Florida approved these experiments. After animals were euthanized, the diaphragm was removed and the crural portion of the diaphragm was removed along with the tendinous attachments.

Address for reprint requests and other correspondence: A. J. Smuder, Dept. of Applied Physiology and Kinesiology, Univ. of Florida, PO Box 118205, Gainesville, FL 32611 (e-mail: asmuder@ufl.edu).
The remaining costal diaphragm was then divided into ipsilateral and contralateral hemidiaphragm sections, and the midcostal region was used for all measurements. The midcostal diaphragm was harvested at the same time post-SCI (36 h) in all experimental groups. The data reported in the main body of the manuscript were obtained by analyzing the midcostal hemidiaphragm ipsilateral to the SCI. We focused on the ipsilateral portion of the diaphragm because it is most directly impacted by the C2 hemileision injury and is likely to be completely paralyzed during the period of 24–36 h postinjury (14, 41). However, the contralateral hemidiaphragm is also impacted by C2 hemileision (14), and accordingly was also evaluated. Due to space limitations, the contralateral diaphragm data are provided in the accompanying Online Supplement. The Online Supplement also contains additional details regarding each of the experimental methods.

**Design: experiment 1.** Animals in experiment 1 were assigned to one of four experimental groups (n = 8/group): 1) acutely anesthetized control (CON), 2) acute spinal cord injury (SCI), 3) 12 h of controlled mechanical ventilation (MV), and 4) acute spinal cord injury with 12 h of controlled mechanical ventilation (SCI + MV).

**Design: experiment 2.** Animals in experiment 2 were assigned to one of three experimental groups (n = 8/group): 1) acute spinal cord injury (SCI), 2) acute spinal cord injury with Trolox treatment (SCI + Trolox), and 3) acute spinal cord injury with 12 h of controlled mechanical ventilation and Trolox treatment (SCI + MV + Trolox).

**SCI surgery.** Rats were anesthetized using inhaled isoflurane (2.5% in oxygen). An 1-in. midline dorsal incision was made over the C1–C4 spinal cord, and the C2 segment was then exposed via laminectomy. The dura was incised and a lateral hemisection lesion was induced on the left side of the spinal cord using a microscalpel and gentle aspiration (9, 11, 23). The dura was closed with interrupted 9-0 sutures and covered with durafilm. The overlying muscle was then sutured in layers and the skin was closed with stainless steel surgical wound clips. After the surgical procedures, buprenorphine (0.03 mg/kg, s.c.) was administered every 12 h for 12 h to provide analgesia.

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**Table 1. Animal heart rate, systolic blood pressure, arterial blood gas tension, and arterial pH during 12 h of mechanical ventilation**

<table>
<thead>
<tr>
<th>Physiological Variable</th>
<th>MV</th>
<th>SCI + MV</th>
<th>SCI + MV + Trolox</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, beats/min</td>
<td>340 ± 8</td>
<td>338 ± 9</td>
<td>324 ± 7</td>
</tr>
<tr>
<td>Systolic blood pressure†, mmHg</td>
<td>116 ± 5</td>
<td>94 ± 6*</td>
<td>91 ± 6*</td>
</tr>
<tr>
<td>Arterial PO2, mmHg</td>
<td>79 ± 4</td>
<td>77 ± 7</td>
<td>72 ± 3</td>
</tr>
<tr>
<td>Arterial PCO2, mmHg</td>
<td>30 ± 2</td>
<td>31 ± 1</td>
<td>32 ± 2</td>
</tr>
<tr>
<td>Arterial pH</td>
<td>7.47 ± 0.01</td>
<td>7.47 ± 0.02</td>
<td>7.49 ± 0.02</td>
</tr>
</tbody>
</table>

Values are means ± SE. Systolic blood pressure values: †at the initiation of mechanical ventilation (MV); ‡average throughout 12 h of MV. SCI, spinal cord injury. *Significantly different vs. control.

**Fig. 1.** Spinal cord injury (SCI) exacerbatates mechanical ventilation (MV)-induced contractile dysfunction and atrophy in the ipsilateral hemidiaphragm. In vitro diaphragm force-frequency response (A) and cross-sectional area of diaphragm skeletal muscle myofibers (B) expressing myosin heavy chain (MHC) I (Type I), MHC IIa (Type IIa), and MHC IIb/IIX (Type IIb/IIX). Representative fluorescent staining of MHC I (DAPI filter/blue), MHC IIa (FITC filter/green), and dystrophin (Rhodamine filter/red) proteins in diaphragm samples are shown below the graph. Values are means ± SE. §Control (CON) significantly different vs. MV and SCI. ¶SCI + MV significantly different vs. all groups. *Significantly different vs. CON (P < 0.05).
mg/kg sc, Hospira, IL) and lactated Ringer solution (5 ml sc) were administered.

Mechanical ventilation. Animals in the MV groups were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg body wt), tracheostomized, and mechanically ventilated with a pressure-controlled ventilator (Servo Ventilator 300, Siemens) for 12 h with the following settings: upper airway pressure limit: 10 cm H2O; respiratory rate: 80 breaths/min; PEEP: 1 cm H2O (34). A venous catheter was inserted into the jugular vein for continuous infusion of pentobarbital sodium (~10 mg·kg⁻¹·h⁻¹). The carotid artery was cannulated to permit the continuous measurement of systolic blood pressure and the collection of blood during the protocol. Fluid balance was maintained via intravenous administration of saline (~2 mg·kg⁻¹·h⁻¹).

Control animals were acutely anesthetized with pentobarbital sodium (60 mg/kg body wt ip). Their diaphragms were quickly removed and stored for subsequent analysis.

Trolox administration. Trolox (Sigma Aldrich) was administered in select groups at 20 mg/kg (ip) at the time of SCI and every 12 h until euthanasia. During MV Trolox was given as previously described (3, 27, 55). All animals were euthanized 36 h after SCI.

In vitro diaphragm contractile properties. Upon euthanasia, a diaphragm muscle strip was dissected from the midcostal region. The strip was suspended vertically with one end connected to an isotonic force transducer (model 6350, Aurora Scientific, Ontario, Canada) within a jacketed bath. Diaphragm contractile properties were measured as described (34).

Diaphragm cross-sectional area and fiber type. Immunohistochemistry was performed to identify muscle fiber types and fiber cross-sectional area (CSA) was determined as described (34).

Mitochondrial respiration and ROS production. Mitochondrial oxygen consumption was measured in permeabilized diaphragm fiber bundles as previously described (21). The maximal respiration (state 3) and basal respiration (state 4) were measured as described previously (10). The respiratory control ratio (RCR) was calculated by dividing state 3 by state 4 respiration. Diaphragm mitochondrial ROS emission was determined using Amplex Red (Molecular Probes, Eugene, OR) as previously described (8).

Western blot analysis. Diaphragm protein extracts were assayed as previously described (34). LC3B (Cell Signaling, Boston, MA), Spec- trin, α-Jun-N-terminal kinase (JNK), phosphorylated JNK (pJNK), extracellular-regulated kinase 1/2 (ERK), phosphorylated ERK (pERK) (Santa Cruz Biotechnology, Santa Cruz, CA), Mus, Agrin, LRP4, 4-hydroxynonenal (4-HNE), and Fn14 (Abcam, Cambridge, MA) were measured. Finally, α-tubulin (Santa Cruz) was used as a loading control for all Western blots.

Real-time polymerase chain reaction. One microliter of cDNA was added to a 25 μl PCR reaction for real-time PCR using Taqman chemistry and the Applied Biosystems (ABI) Prism 7000 Sequence Detection system (ABI, Foster City, CA) as previously described (8).

### Table 2. Mitochondrial oxygen consumption in permeabilized ipsilateral diaphragm muscle fibers

<table>
<thead>
<tr>
<th>Treatment</th>
<th>State 3</th>
<th>State 4</th>
<th>RCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>13.16 ± 1.11</td>
<td>3.04 ± 0.97</td>
<td>7.98 ± 0.65</td>
</tr>
<tr>
<td>SCI</td>
<td>19.45 ± 4.15</td>
<td>6.70 ± 1.38*</td>
<td>3.01 ± 0.15*</td>
</tr>
<tr>
<td>MV</td>
<td>11.10 ± 0.77</td>
<td>3.66 ± 0.19</td>
<td>4.44 ± 0.27*</td>
</tr>
<tr>
<td>SCI + MV</td>
<td>14.40 ± 1.91</td>
<td>4.84 ± 0.66</td>
<td>3.23 ± 0.14†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 8/group. These data were obtained using pyruvate/malate as the substrate. Units for state 3 and state 4 are nanomoles of oxygen per milligram of tissue (dry weight) per minute. RCR, respiratory control ratio. *Significantly different vs. control (CON); †significantly different vs. CON and MV (P < 0.05).

### RESULTS

#### Systemic and Biologic Response to MV

Body weight was no different between the experimental groups prior to the interventions (experiment 1: CON 278 ± 8 g, SCI 287 ± 3 g, MV 291 ± 9 g, SCI + MV 289 ± 8 g; experiment 2: SCI 277 ± 4 g, SCI + Trolox 285 ± 8 g, SCI + MV + Trolox 292 ± 9 g). Heart rate (HR), systolic blood pressure (SBP), arterial partial pressures of O2 (PaO2), CO2 (PaCO2), and pH were all maintained relatively constant during MV (Table 1). Average and initial SBP were reduced in mechanically ventilated rats with SCI. However, impaired ability to regulate blood pressure is a typical complication in SCI patients (33). Colonic (body) temperature was maintained at 36°C–37°C during MV. At the completion of MV, there was no visible indication of lung injury or infection.

#### Statistical Analyses

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

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**Fig. 2.** SCI and MV increase oxidative stress in the ipsilateral hemidiaphragm. 

A: mitochondrial hydrogen peroxide emission from permeabilized diaphragm muscle fibers using pyruvate/malate as the substrate. Values are normalized to tissue dry weight. B: Western blot of 4-HNE modified proteins measured in whole diaphragm muscle normalized to tubulin. Representative Western blot images for 4-HNE and tubulin are shown to the right of the graph. Values are means ± SE. §§Significantly different vs. all groups. *Significantly different vs. CON (P < 0.05).
Experiment 1

Cervical SCI exacerbates VIDD. The in vitro force-frequency response was evaluated in strips of midcostal diaphragm muscle. In the ipsilateral diaphragm, specific force production was significantly reduced in both the MV and SCI groups. However, the combination of SCI + MV further reduced diaphragm force production compared with either treatment alone (Fig. 1A). All experimental groups showed a reduction in ipsilateral hemidiaphragm myofiber CSA for type I and type IIb/x fibers compared with control (Fig. 1B). Only the combination of SCI + MV resulted in reductions in type IIa fiber CSA vs. control. SCI + MV also further reduced type IIb/x myofiber CSA compared with either condition alone.

Force production and diaphragm muscle CSA in the contralateral hemidiaphragm were diminished in both the MV and SCI + MV groups compared with control, and SCI + MV also resulted in decreased contralateral diaphragm force production and diaphragm CSA compared with SCI alone (Supplemental Fig. S1).

SCI and MV increase mitochondrial dysfunction. Inactivity-induced skeletal muscle atrophy occurs as a result of mitochondrial dysfunction, which results in increased ROS production and oxidative stress within muscle fibers (21, 36). In the ipsilateral hemidiaphragm, mitochondrial RCR was reduced in all groups compared with control, and the SCI + H11001 MV group demonstrated a further reduction compared with MV (Table 2). Mitochondrial hydrogen peroxide emission was also increased in all experimental groups compared with control, and ROS production was further elevated in SCI + MV tissues compared with either condition alone (Fig. 2A). Furthermore, lipid peroxidation was increased in all groups compared with control (Fig. 2B).

Mitochondrial RCR in the contralateral hemidiaphragm was reduced in both the MV and SCI + MV groups compared with control or SCI (Supplemental Table S1). Mitochondrial ROS emission in the contralateral diaphragm was elevated in the MV animals compared with control, and in the SCI + MV group, ROS was elevated compared with all groups (Supplemental Fig. S2A). Finally, 4-HNE modified proteins were increased in both MV and SCI + MV compared with control and SCI (Supplemental Fig. S2B).

SCI and MV increase proteolytic activity. The ubiquitin-proteasome and autophagy/lysosomal systems are two major proteolytic systems responsible for the removal of proteins. The activity of these two systems is upregulated during prolonged periods of skeletal muscle inactivity (15, 19, 32, 50). Here we found that ipsilateral hemidiaphragm mRNA expression of the E3 ligases Atrogin-1/MaFbx and MuRF1 was upregulated in all experimental groups compared with control. Similar increases in Atrogin-1/MaFbx mRNA expression were observed across treatment groups, but the combination of SCI + MV resulted in a unique increase in mRNA expression of MuRF1.
Fig. 4. SCI augments Fn14 expression and signaling in the ipsilateral hemidiaphragm. A: real-time RT-PCR analysis for Fn14. Gene expression is presented as corrected for β-glucoronidase and normalized to control values. B: Western blot analysis of Fn14. C: Western blot analysis of the ratio of pJNK/JNK. D: Western blot analysis of the ratio of pERK/ERK. All Western blot analyses are presented as corrected for tubulin. Representative images are shown to the right of the graph. Values are means ± SE. †Significantly different vs. CON and MV. *Significantly different vs. CON (P < 0.05).

Fig. 5. SCI and MV alter neuromuscular junction protein expression in the ipsilateral hemidiaphragm. A: real-time RT-PCR analysis for MuSK, Agrin, and LRP4. Gene expression is presented as corrected for β-glucoronidase and normalized to control values. B: Western blot analysis of MuSK. C: Western blot analysis of Agrin. D: Western blot analysis of LRP4. All Western blot analyses are presented as corrected for tubulin. Representative images are shown to the right of the graph. Values are means ± SE. *Significantly different vs. CON (P < 0.05).
compared with all groups (Fig. 3A). In contrast, significant increases in Atrogin-1/MaFbx and MuRF1 were only demonstrated in the MV and SCI + MV groups in the contralateral hemidiaphragm (Supplemental Fig. S3A).

LC3 is required in the formation of autophagosomes, and we found an increase in the mRNA expression of LC3 as well as the ratio of LC3II/LC3I in the ipsilateral (Fig. 3, A and B) and contralateral (Supplemental Fig. S3, A and B) hemidiaphragm in all experimental groups compared with control.

Finally, calpain and caspase-3 are two proteases capable of cleaving intact myofibrillar proteins resulting in skeletal muscle weakness (29, 51). Cleavage of α-II-spectrin provides an index of calpain and caspase-3 proteolytic activity (29, 51, 54). Increases in the 145-kDa fragment of α-II-spectrin are indicative of increased calpain activity, and increases in the 120-kDa fragment indicate caspase-3 activity. Both fragments were increased in the ipsilateral hemidiaphragm in all experimental groups compared with control (Fig. 3, C and D). In the contralateral hemidiaphragm, SCI alone did not increase either fragment. However, calpain and caspase-3 activity were elevated in the MV and SCI + MV groups (Supplemental Fig. S3, C and D).

SCI and MV upregulate TWEAK/Fn14. Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) through binding to its receptor fibroblast growth factor inducible 14 (Fn14/TWEAKR) can promote skeletal muscle atrophy (42). SCI and SCI + MV resulted in increased Fn14 mRNA and protein expression (Fig. 4, A and B) in the ipsilateral hemidiaphragm compared with control and MV. TWEAK/Fn14-mediated signaling can phosphorylate both ERK and JNK (42). We also found increases in JNK phosphorylation in SCI and SCI + MV groups compared with both control and MV (Fig. 4C). Ipsilateral hemidiaphragm ERK phosphorylation was influenced by both injury and ventilation with increased levels in both MV and SCI + MV animals compared with control (Fig. 4D).

In the contralateral hemidiaphragm, mRNA expression of TWEAK and the ratio of pJNK/JNK were similarly increased in all experimental groups compared with control (Supplemental Fig. S4, A and C). Protein expression of Fn14 was elevated in MV animals compared with control and in SCI + MV animals compared with all groups (Supplemental Fig. S4B). No differences were detected in the ratio of pERK/ERK (Supplemental Fig. S4D).

SCI and MV alter neuromuscular junction protein expression. Agrin is a nerve-derived organizer of postsynaptic differentiation during neuromuscular junction (NMJ)
Table 3. **Mitochondrial oxygen consumption in permeabilized ipsilateral diaphragm muscle fibers**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>State 3</th>
<th>State 4</th>
<th>RCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCI</td>
<td>20.69 ± 3.87</td>
<td>5.94 ± 1.28</td>
<td>3.82 ± 0.20</td>
</tr>
<tr>
<td>SCI + Trolox</td>
<td>12.07 ± 0.61</td>
<td>2.96 ± 0.17</td>
<td>5.32 ± 0.47</td>
</tr>
<tr>
<td>SCI + MV + Trolox</td>
<td>9.28 ± 0.51#</td>
<td>2.44 ± 0.19#</td>
<td>4.86 ± 0.24</td>
</tr>
</tbody>
</table>

Values are mean ± SE; n = 8/group. These data were obtained using pyruvate/malate as the substrate. Units for state 3 and state 4 are nanomoles of oxygen per milligram of tissue (dry weight) per minute. #Significantly different vs. SCI (P < 0.05).

formation (58). SCI + MV causes a significant reduction in ipsilateral Agrin mRNA expression (Fig. 5A), but trends for differences in protein expression across groups did not reach statistical significance (Fig. 5C). No differences in Agrin mRNA expression were detected in the contralateral hemidiaphragm, but a reduction in Agrin protein expression was detected in the SCI group compared with control (Supplemental Fig. S5, A and C).

MuSK is a receptor tyrosine kinase that is essential for Agrin-induced clustering and for NMJ formation (58). In the ipsilateral hemidiaphragm, providing MV support after SCI induced an increase in both mRNA and protein expression of MuSK compared with CON (Fig. 5, A and B). In the contralateral hemidiaphragm mRNA expression was similar across all experimental groups, but MuSK protein expression was increased in the MV and SCI + MV groups compared with control and SCI (Supplemental Fig. S5, A and B).

The transmembrane protein LRP4 is required for Agrin-mediated activation of MuSK (58). We found no differences in LRP4 mRNA or protein expression across any group (Fig. 5, A and D; Supplemental Fig. S5, A and D).

**Experiment 2**

**Trolox administration prevents SCI-induced diaphragm dysfunction.** Oxidative modification of muscle contractile proteins can result in contractile dysfunction and accelerate protein breakdown resulting in atrophy of skeletal muscle fibers (3, 36). Here we found that treatment with the antioxidant Trolox significantly increased ipsilateral hemidiaphragm specific force production in both the SCI and the SCI + MV groups compared with SCI rats that did not receive Trolox (Fig. 6A). The increases in specific force production were evident at stimulation frequencies greater than 30 Hz (Fig. 6A). Trolox also prevented the reductions in ipsilateral diaphragm type I and type IIb/x myofiber CSA that occurred after SCI (Fig. 6B). In the contralateral hemidiaphragm, Trolox treatment prevented SCI-induced decreases in diaphragm force at stimulation frequencies greater than 60 Hz (Supplemental Fig. S6A). No differences in CSA existed for any diaphragm fiber type across the experimental groups (Supplemental Fig. S6B).

**Trolox administration reduces mitochondrial damage and ROS production.** Trolox protected against the SCI-induced reductions in RCR (Table 3) and also reduced mitochondrial hydrogen peroxide emission in the ipsilateral hemidiaphragm (Fig. 7A). The accumulation of 4-HNE modified proteins was attenuated in both the SCI and SCI + MV experimental groups following Trolox treatment (Fig. 7B). In the contralateral hemidiaphragm, no differences existed in mitochondria function or oxidative damage between groups (Supplemental Table S2 and Supplemental Fig. S7, A and B).

**Trolox reduces diaphragm proteolysis.** Both Atrogin-1/MAFBx and MuRF1 mRNA expression were reduced in the ipsilateral hemidiaphragm in SCI + Trolox and SCI + MV + Trolox animals compared with SCI alone (Fig. 8A). Trolox treatment also reduced the mRNA expression of LC3, the ratio of LC3II/LC3I, and reduced the accumulation of the 145-kDa spectrin fragment in both Trolox-treated groups compared with SCI alone (Fig. 8, A–C). No differences in proteolytic activity were detected between experimental groups in the contralateral hemidiaphragm (Supplemental Fig. S8, A–D).

**Trolox attenuates SCI-induced TWEAK signaling.** Administration of Trolox to SCI and SCI + MV rats reduced mRNA expression of Fn14 in the ipsilateral hemidiaphragm (Fig. 9A). In addition, Fn14 protein expression was reduced in SCI + MV + Trolox animals compared with SCI and SCI + Trolox (Fig. 9B). While Trolox had no effect on the pJNK/JNK ratio, the ratio of pERK/ERK was reduced after Trolox in all experimental groups compared with SCI alone (Fig. 9, C and D). Trolox also reduced mRNA expression of Fn14 in the contralateral hemidiaphragm of all experimental groups compared...
with SCI (Supplemental Fig. S9A). However, no differences were detected in contralateral hemidiaphragm protein expression of Fn14 or in the ratio of pJNK/JNK and pERK/ERK (Supplemental Fig. S9, B–D).

Trolox does not affect the expression of NMJ proteins. Protein and mRNA expression of the NMJ proteins MuSK, Agrin, and LRP4 were not statistically affected by Trolox in the ipsilateral or contralateral hemidiaphragm (Fig. 10, A–D; Supplemental Fig. S10, A–D). However, there was a clear tendency for a reduction in MuSK protein levels in the ipsilateral hemidiaphragm in the groups receiving Trolox (Fig. 10B, P = 0.1044).

DISCUSSION

Respiratory complications are a hallmark of acute SCI and necessitate MV in approximately two-thirds of patients (4, 46). The extent of respiratory dysfunction correlates with the degree and segmental level of the injury, with high cervical SCI having the greatest risk of MV dependency (7, 12). Our study reveals that acute cervical SCI exacerbates the negative impact of MV on the diaphragm, further reducing myofiber size and contractile function. Hence, providing ventilator support rapidly after SCI exacerbates VIDD, which is likely to complicate weaning from MV independent of neurological considerations. Our findings also provide the first evidence that antioxidant therapies may prove beneficial for minimizing VIDD after SCI since Trolox administration attenuated the negative cumulative impact of SCI and MV on diaphragm function.

ROS Production After SCI and MV

VIDD occurs, at least in part, due to elevated ROS production, which promotes increased oxidative damage and accelerated protein breakdown in the diaphragm (21). Our group has demonstrated that in rats, MV-induced oxidative injury in the diaphragm does not occur immediately after the onset of MV. However, significant increases in protein carbonyls and lipid hydroperoxide levels are present as quickly as 6 h after the initiation of MV, which is consistent with the initiation of proteolytic activity in the diaphragm (28, 59). Disruption of phrenic output to the diaphragm also increases lipid peroxidation and decreases antioxidant enzyme activity, which may contribute to SCI-induced diaphragm dysfunction (43). Our results confirm that MV induces the elevation of mitochondrial ROS emission and lipid peroxidation, and further show that similar processes are triggered by cervical SCI. More importantly, compared with SCI or MV alone, we observed that mitochondrial ROS emission was exacerbated when MV sup-
port was provided acutely following SCI. This finding is consistent with the concept that oxidative stress is a fundamental component of the mechanism(s) that lead to the additive effects of MV on SCI-induced diaphragm dysfunction.

Increased ROS emission enhances myofiber protein degradation via activation of several proteolytic systems including caspase-3, calpain, the ubiquitin-proteasome system, and the autophagy/lysosomal system (16, 34, 49, 56). Data from both human and animal experiments confirm that these proteolytic systems are activated in the diaphragm following MV (19, 24, 34). Additionally, cervical SCI increases diaphragm mRNA expression of key proteins of the ubiquitin-proteasome system, and the caspase-3 and calpain proteolytic pathways (14). Thus both MV and cervical SCI activate similar proteolytic pathways in the diaphragm. Importantly, the current experiments reveal that providing MV support following cervical SCI leads to an additive increase in MuRF1 expression. Thus this essential mediator of skeletal muscle atrophy (15) could play a role in enhancing VIDD following SCI.

**TWEAK/Fn14 Signaling**

TWEAK is a member of the TNF superfamily of cytokines, and increased expression of TWEAK and/or its receptor (Fn14) is linked to denervation-induced skeletal muscle breakdown (42). Specifically, following skeletal muscle denervation increased expression of Fn14 facilitates TWEAK-induced activation of NF-κB transcriptional activity, which subsequently increases the gene expression of MuRF1 (42). The enhanced expression of Fn14 in the diaphragm after cervical SCI indicates that the TWEAK/Fn14 system could contribute to the increased expression of MuRF1 in the diaphragm after MV and cervical SCI. Finally, activation of the MAPKs ERK and JNK plays a critical role in the induction of Fn14 expression in denervated skeletal muscle (52). Our results are consistent with this finding, as cervical SCI resulted in increased phosphorylation of JNK and ERK in the diaphragm.

**The Phrenic-Diaphragm NMJ**

Morphological adaptations to the phrenic-diaphragm NMJ after cervical SCI include both expansion and elongation of terminal phrenic axonal branches (14, 37). Additionally, Nicaise et al. (30) demonstrated that contusion injury at the C4 and C5 levels resulted in diaphragm dysfunction as a result of phrenic motor neuron loss, phrenic nerve axonal degeneration, and denervation at diaphragm NMJs (30). Cervical SCI also results in altered expression of genes associated with the NMJ including MuSK and agrin (14). Under normal conditions, motor neurons release agrin that binds to and activates LRP4 in muscle cells, stimulating MuSK kinase activity. Indeed, the agrin/LRP4/MuSK pathway is essential for survival, and genetic deletion of any of these proteins results in respiratory failure (18, 38, 39). Our results suggest that MV and cervical SCI result in alterations to NMJ function because both MV and SCI resulted in elevated mRNA expression of MuSK and
reduced expression of agrin in the diaphragm. These changes may reflect a compensatory mechanism to improve neuromuscular transmission during conditions where the diaphragm is inactive.

Antioxidant Therapy Prevents VIDD After Cervical SCI

The vitamin E analog Trolox protects against oxidative stress through its radical scavenging activity (17). Specifically, Trolox administration can eliminate intracellular ROS and suppress oxidative stress (17, 40). Decreased levels of intracellular ROS diminish the amount of oxidatively damaged biomolecules, which in turn reduces the activation of calpain, caspase-3, the ubiquitin-proteasome system and the autophagy/lysosomal system (27, 53, 55). These proteolytic systems are all sensitive to oxidative damage, and previous reports demonstrate that Trolox administration during MV reduces oxidative damage, increases proteolysis of the diaphragm, and protects against VIDD (27, 53, 55). Our results agree with these previous findings and confirm that SCI-induced diaphragm atrophy can also be prevented by Trolox administration. Therefore, antioxidant administration is a potential therapeutic intervention to combat weaning problems induced by the combined effects of MV on acute SCI-induced diaphragm dysfunction.

The Response of Diaphragm Myofibers Contralateral to the Cervical SCI

The primary goal in focusing on the lateral C2 hemisection model was to stimulate the condition of acute diaphragm paralysis and MV support. While the hemidiaphragm ipsilateral to the hemisection will be inactive during the 0–36 h postinjury time period, the contralateral diaphragm is also influenced by the C2 hemisection injury and can also have a compensatory increase in myofiber discharge (41). It is interesting, therefore, to contrast the response of the ipsilateral (Figs. 1–10) vs. contralateral hemidiaphragm (Supplemental Data) to SCI, as well as the combination of SCI and MV. When assessed 36 h post-SCI, the contralateral diaphragm showed a moderate decline in contractile force but with no significant changes in myofiber CSA (Supplemental Fig. S1). Similar to a prior report (14), the contralateral diaphragm showed a tendency for increased expression of atrophy-related genes including Atrogin-1/MaFbx, MuRF1, and LC3 (Supplemental Fig. S2). Thus the spinal lesion impacted contralateral diaphragm myofibers, but the observed changes are unlikely to reflect processes associated with myofiber inactivity since the contralateral diaphragm remains active. Thus nonspecific processes (e.g., systemic inflammation) are likely to contribute to atrophy and/or dysfunction of contralateral

Fig. 10. Trolox does not affect the expression of neuromuscular junction proteins in the ipsilateral hemidiaphragm. A: real-time RT-PCR analysis for MuSK, Agrin, and LRP4. Gene expression is presented as corrected for β-glucoronidase and normalized to control values. Dotted line represents control values. B: Western blot analysis of MuSK. C: Western blot analysis of Agrin. D: Western blot analysis of LRP4. All Western blot analyses are presented as corrected for tubulin. Representative images are shown to the right of the graph. Values are means ± SE.
diaphragm myofibers acutely after SCI (14). Finally, in
regards to the contratralateral diaphragm, we also observed
that the combination of SCI and MV leads to the greatest
decline in contractile force (Supplemental Fig. S1), and that
Trolox was effective at mitigating this response (Supple-
mental Fig. S6). Thus the functional benefits of antioxidant
treatment were not exclusive to the inactive (denervated)
motor units, but could also be observed in myofibers con-
tratralateral to the injury.

Conclusions

It is well established that VIDD is a prevalent complication
of prolonged MV and that VIDD is a potential contributor to
difficult weaning from the ventilator (25, 35, 45). The present
study confirmed that cervical SCI leads to diaphragm atrophy
dysfunction (14, 26, 30). More importantly, using an
animal model of MV, our results conclusively show that SCI
creates preconditions that greatly exacerbate VIDD. Accord-
ingly, VIDD will likely be increased when ventilator support
is provided after SCI, and independent of neurological consider-
ations, diaphragm dysfunction is likely to complicate weaning
from MV. Finally, our findings highlight the importance of
developing strategies to minimize VIDD. In this regard, anti-
oxidant therapy may be useful to ameliorate diaphragm dys-
function resulting from cervical SCI alone or from the combi-
nation of cervical SCI and MV.

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AUTHOR CONTRIBUTIONS

design of research; A.J.S., E.J.G.-R., O.S.K., A.B.M., and K.K.S. performed
treatment were not exclusive to the inactive (denervated)

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