Mechanical ventilation promotes redox status alterations in the diaphragm


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Falk, D. J., K. C. DeRuisseau, D. L. Van Gammeren, M. A. Deering, A. N. Kavazis, and S. K. Powers. Mechanical ventilation promotes redox status alterations in the diaphragm. J Appl Physiol 101: 1017–1024, 2006. First published May 4, 2006; doi:10.1152/japplphysiol.00104.2006.—Oxidative stress is an important mediator of diaphragm muscle atrophy and contractile dysfunction during prolonged periods of controlled mechanical ventilation (MV). To date, specific details related to the impact of MV on diaphragmatic redox status remain unknown. To fill this void, we tested the hypothesis that MV-induced diaphragmatic oxidative stress is the consequence of both an elevation in intracellular oxidant production in conjunction with a decrease in the antioxidant buffering capacity. Adult rats were assigned to one of two experimental groups: 1) control or 2) 12 h of MV. Compared with controls, diaphragms from MV animals demonstrated increased oxidant production, diminished total antioxidant capacity, and decreased glutathione levels. Heme oxygenase-1 (HO-1) mRNA and protein levels increased (23.0- and 5.1-fold, respectively) following MV. Thioredoxin reductase-1 and manganese superoxide dismutase mRNA levels were also increased in the diaphragm following MV (2.4- and 1.6-fold, respectively), although no change was detected in the levels of either protein. Furthermore, copper-zinc superoxide dismutase and glutathione peroxidase mRNA were not altered following MV, although protein content decreased ∼1.3- and ∼1.7-fold, respectively. We conclude that MV promotes increased oxidant production and impairment of key antioxidant defenses in the diaphragm; collectively, these changes contribute to the MV-induced oxidative stress in this key inspiratory muscle.

Muscle wasting; atrophy; oxidative stress; superoxide dismutase; catalase; glutathione; heme oxygenase; thioredoxin reductase

It is well established that extended periods of skeletal muscle disuse lead to the development of muscle atrophy (12, 14, 16, 25, 26, 36). Conditions such as limb immobilization, prolonged bed rest, and spaceflight illicit atrophy of locomotor skeletal muscle (6, 7, 18). Moreover, recent evidence reveals that short periods of diaphragmatic inactivity (e.g., 18 h), induced by mechanical ventilation, also result in atrophy of this important inspiratory muscle (5, 19, 29, 33, 45). A common denominator between all of the models of disuse skeletal muscle atrophy is that oxidative stress plays a pivotal role in the development of atrophy, contractile dysfunction, and increased proteolysis (9, 13, 18, 20, 28).

Mechanical ventilation-induced diaphragmatic atrophy is a unique and clinically important type of disuse skeletal muscle atrophy. In medicine, mechanical ventilation is used to maintain adequate alveolar ventilation in patients that cannot do so on their own. Although several modes of mechanical ventilation are used clinically, controlled mechanical ventilation (MV) is the mode whereby all breaths are delivered by the ventilator. During controlled MV, the diaphragm is mechanically inactive and rapidly develops atrophy and oxidative injury (5, 9, 35).

Although all forms of disuse muscle atrophy have clinical significance, the diaphragmatic atrophy and contractile dysfunction resulting from MV is of particular importance given that respiratory muscle atrophy and weakness is an important contributor to the failure to wean patients from mechanical ventilation (11, 15, 38, 41). In reference to MV and diaphragmatic oxidative injury, our laboratory has shown that diaphragmatic unloading is associated with a rapid onset of diaphragmatic oxidative stress that develops within 3–6 h after initiating MV (43). Importantly, administration of the water-soluble vitamin E analog Trolox attenuates the MV-induced oxidative stress, retards muscle proteolysis, and prevents MV-induced diaphragmatic contractile dysfunction (5). Hence, oxidative stress plays an important role in MV-induced diaphragm contractile dysfunction and atrophy. At present, it is currently unknown whether MV-induced oxidative stress in the diaphragm is the result of an increase in oxidant production and/or impaired antioxidant capacity. Therefore, these experiments determined the effects of MV on diaphragmatic oxidant production and total antioxidant capacity, and mRNA and protein levels of key antioxidant enzymes. We hypothesized that the development of MV-induced diaphragmatic oxidative stress is the consequence of both increased intracellular oxidant production combined with a decrease in the antioxidant-buffering capacity.

Materials and Methods

Animals. Female, 6-mo-old Sprague-Dawley rats (Harlan County, IN) were randomly assigned to one of two experimental groups: 1) acutely anesthetized control (n = 11), or 2) 12 h of MV (n = 11). The Animal Care and Use Committee of the University of Florida approved these experiments.

Experimental protocol. Animals in the control group were acutely anesthetized with pentobarbital sodium (60 mg/kg ip). After reaching a surgical plane of anesthesia, the control animals were killed immediately and diaphragms were quickly removed. Approximately one-third of the medial costal diaphragm was used for immediate ex vivo measurement of intracellular oxidant production using dichlorofluorescein (DCFH), and the remaining portion of the costal diaphragm was stored at −80°C for subsequent analyses.

Animals in the MV group were anesthetized with pentobarbital sodium (60 mg/kg ip). After reaching a surgical plane of anesthesia, the animals were tracheostomized using aseptic techniques and mechanically ventilated with a controlled volume-driven ventilator (Inspira, Harvard Apparatus, Cambridge, MA) for 12 h (tidal volume: ∼0.55 ml/100 g; respiratory rate: 80 breaths/min; positive end-expiratory pressure: 1 cmH2O). We chose 12 h of MV because...
this duration is associated with diaphragmatic contractile dysfunction, increased rates of proteolysis, and oxidative stress.

The carotid artery was cannulated to permit continuous measurement of blood pressure, saline infusion, and the collection of arterial blood samples during the first and last hour of the experiment. Previous experiments in our laboratory have shown that our mechanical ventilation protocol is successful in maintaining blood-gas and pH homeostasis in animals for periods of 6–18 h (8, 29, 35, 43). In the current experiments, blood samples were periodically obtained and analyzed for pH and the partial pressures of O$_2$ and CO$_2$ using an electronic blood-gas analyzer (GEM Premier 3000; Instrumentation Laboratory, Lexington, MA). If necessary, adjustments were made to the ventilator volume to ensure that arterial blood-gas and pH measures were within the desired ranges. Arterial Po$_2$ was maintained >70 Torr throughout the experiment by adjustments in FIO$_2$ (22–25% oxygen).

The jugular vein was cannulated for the constant infusion of pentobarbital sodium (−10 mg·kg$^{-1}$·h$^{-1}$). Body temperature was maintained at 37°C, 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, and electrocardiograph. Continuous care during the MV protocol included maintenance at 37°C, 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, and expressing the bladder, removing airway mucus. The carotid artery was cannulated to permit continuous measure-ment of blood pressure, saline infusion, and the collection of arterial blood samples during the first and last hour of the experiment. Previous experiments in our laboratory have shown that our mechanical ventilation protocol is successful in maintaining blood-gas and pH homeostasis in animals for periods of 6–18 h (8, 29, 35, 43). In the current experiments, blood samples were periodically obtained and analyzed for pH and the partial pressures of O$_2$ and CO$_2$ using an electronic blood-gas analyzer (GEM Premier 3000; Instrumentation Laboratory, Lexington, MA). If necessary, adjustments were made to the ventilator volume to ensure that arterial blood-gas and pH measures were within the desired ranges. Arterial Po$_2$ was maintained >70 Torr throughout the experiment by adjustments in FIO$_2$ (22–25% oxygen).

The jugular vein was cannulated for the constant infusion of pentobarbital sodium (−10 mg·kg$^{-1}$·h$^{-1}$). Body temperature was maintained at 37°C, and heart rate was monitored via a lead II electrocardiograph. Continuous care during the MV protocol included lubricating the eyes, rotating the animal, passive limb movement, and enteral nutrition (Research Diets, New Brunswick, NJ). Animals also received an intramuscular injection of glycopyrrolate (0.04 mg/kg) every 2 h during MV to avoid airway secretions. Following the experimental protocol, the diaphragm was quickly removed, and approximately one-third of the medial costal diaphragm was used for immediate ex vivo measurement of intracellular oxidant production using 2',7'-dichlorofluorescein (DCFH) and the remaining portion of the costal diaphragm was stored at −80°C for subsequent analyses.

Measurement of intracellular oxidant production with DCFH. To assess intracellular oxidant production of diaphragm muscle fiber bundles, detection of DCFH oxidation was performed according to the procedures outlined by Arborgast and Reid (1) with minor modifications. Briefly, costal diaphragm strips (~40 mg) were isolated and subsequently incubated at an unstressed (resting) length for 45 min in the dark in a Krebs solution (pH 7.4) maintained at 25°C, equilibrated with a 95% O$_2$-5% CO$_2$ gas mixture that contained 50 μM 2',7'-dichlorofluorescein diacetate (DCFH-DA) protocol included rinsing in Krebs solution (without DCFH-DA), muscle fiber bundles were fixed to a coverslip at resting length and images were immediately obtained with an inverted fluorescent microscope (Carl Zeiss Axiovert 200) using a 480-nm low-pass excitation filter and 520-nm high-pass emissions filter. Light was filtered using a 5% neutral density filter, and DCF emissions were recorded using a charge-coupled device camera (Retiga QImaging-EXi). Three to five images were obtained along the fiber bundle with care taken to avoid repeated exposure.

Acquisition and analysis of images obtained were performed using commercial image analysis software (IPLAB v3.5, Scionalytics).

Total antioxidant capacity. To determine the representative level of total diaphragmatic antioxidants, we used a modification of the technique described by Re et al. (30) and Lawler and colleagues (18). This procedure effectively measures the capacity of skeletal muscle homogenate to scavenge the blue/green chromophore ABTS$^+$ (2',7'-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid; Sigma Chemical, St. Louis, MO). The scavenging capacity of the homogenate is calculated as the rate of decolorization expressed as a percentage of the inhibition of ABTS$^+$ color change measured at 734 nm. This is compared with a Trolox standard curve under identical conditions. This assay provides an indication of skeletal nonenzymatic antioxidant scavengers (i.e., ascorbate, reduced glutathione, β-carotene, uric acid, α-tocopherol, bilirubin). ABTS$^+$ was activated by reaction of ABTS chemical (7 mM) with 2.5 mM potassium persulfate (Aldrich, Milwaukee, WI) in 100 mM potassium phosphate buffer (pH = 7.4). Note that the resulting radical cation is stable in the dark at 4°C for 1 wk. Nonetheless, in the current experiments, ABTS$^+$ was prepared 24 h before use in the assay and was diluted (~70:1) to an absorbance of 0.70 ± 0.02 at 734 nm. After dilution, 270 μl of ABTS$^+$ solution were added to each well of the microplate. The initial absorbance at 734 nm was recorded. Then 20 μl of Trolox or sample were added to each well and the absorbance was recorded for 5 min. Parallel blanks were run with the buffer alone. The decolorization of ABTS$^+$, as determined through a reduction in absorbance, was a function of antioxidant concentration and equated relative to the reactivity of Trolox. Total antioxidant capacity (TAC) for each sample was then calculated as micromoles of Trolox equivalent per gram of tissue.

Isolation of total RNA. A portion of the costal diaphragm (~60 mg) was homogenized in 1.5 ml of Trizol reagent (Invitrogen, Carlsbad, CA) and processed according to the manufacturer’s instructions. Samples were homogenized with a Polytron blade homogenizer at medium speed and centrifuged at 12,000 g for 10 min (4°C) to remove insoluble material. The RNA portion was transferred to a new tube and extracted with 120 μl of phenol-chloroform. Samples were vortexed briefly and centrifuged at 13,000 g for 20 min (4°C). After transfer of the aqueous phase to a new tube, RNA was precipitated with one volume of isopropanol and washed twice with two volumes of 75% ethanol. The RNA was pelleted via centrifugation and resuspended in RNAse-free water (Sigma). Concentration and purity of the extracted RNA was measured spectrophotometrically at 260 nm and at 280 nm in 1× TE buffer (10 mM Tris·HCl, pH 8.0, 1 mM EDTA) (Promeza, Madison, WI). The integrity of the extracted total RNA was verified by gel electrophoresis of 1 μg RNA on a 1% agarose ethidium bromide-stained (89 mM Tris, 89 mM boric acid, 2 mM EDTA) TBE gel. Total RNA was stored at −80°C.

Reverse transcription and cDNA quantification. Reverse transcription was performed using the Superscript III First-Strand Synthesis System for RT-PCR (Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions. Reactions were carried out using 5 μg of total RNA and 2.5 μM oligo(DT)$_2$$_2$$_2$$_2$$_2$ primers. First-strand cDNA was subsequently treated with two units of RNase H. After the addition of 2 μl of GlycoBlue coprecipitant (Ambion, Austin, TX) to the RT product (21 μl), first-strand cDNA was purified of RNA and unincorporated nucleotides by treatment with an RNase cocktail (Ambion), brought to a volume of 100 μl with water, and applied to a NucAway spin column (Ambion). Samples were then mixed with phenol:chloroform:isoamyl alcohol (pH = 7.9) and the aqueous phase was recovered using a 1.5 ml heavy phase lock gel (Eppendorf, Hamburg, Germany). The cDNA was precipitated by adding one volume of 5.0 M NH$_4$OAc and two volumes of 100% EtOH then stored at −20°C overnight. After centrifugation at 13,000 g for 20 min (4°C), the cDNA was washed with two volumes of 75% EtOH, centrifuged at 13,000 g for 10 min (4°C), and resuspended in 50 μl of 1× TE buffer.

The cDNA was subsequently quantified using the Oligreen ssDNA Quantiication Reagent and Kit according to the manufacturer’s instructions (Molecular Probes, Eugene, OR).

Real-time quantitative PCR. The probes for all genes consisted of Taqman 5’-labeled FAM reporters and 3’-nonfluorescent quenchers. Quantitative real-time PCR was performed using the ABI Prism 7000 Sequence Detection System (ABI, Foster City, CA). Primers and probes for thioredoxin reductase-1 (TnxRd1), catalase (CAT), glutathione peroxidase (GPX), manganese superoxide dismutase (Mn-SOD), copper-zinc superoxide dismutase (CuZnSOD), and β-glucuronidase were obtained from Applied Biosystems (Assays-on-Demand; ABI). The sequences used by the manufacturer in the design of primers and probes from this service are proprietary and are therefore not reported. The heme oxygenase-1 (HO-1) primers and probes were obtained from Applied Biosystems (Assays-by-Design; ABI). Primer and probe sequences for HO-1 are: forward, 5’-GGTACGTTGACGTTAGGTGTTGAC-3’; reverse, 5’-GCACCAACCTCCCATATTACCTT-3’; probe, 5’-CTTAAAGCTCGGTGATCTACAAC-3’. Each 25 μl PCR reaction, performed in duplicate, contained 3.0 ng of cDNA template. Changes in mRNA expression were calculated using the relative standard curve method as described in the ABI, User Bulletin #2. mRNA input values obtained from standard curves were normal-
ized to β-glucuronidase because this gene did not demonstrate altered expression levels in the diaphragm in response to 12 h of MV (P = 0.99).

Western blot analysis. A section (~50 mg) of the costal diaphragm was homogenized and assayed to quantitatively determine the protein levels of CuZnSOD, MnSOD, CAT, GPX, and TxnRd1. Samples were homogenized 1:10 (wt/vol) in buffer (in mM: 50 NaF, 150 HEPES, 100 NaCl, 10 Na pyrophosphate, 5 EDTA, and Triton X-100 0.5%) with protease inhibitor cocktail (Sigma) and centrifuged at 1,000 g for 30 min at 4°C. After collection of the resulting supernatant, diaphragmatic protein content was assessed by the method of Bradford (Sigma). Proteins (60 μg) were then separated by polyacrylamide gel electrophoresis via 12% gradient polyacrylamide gels containing 0.1% sodium dodecyl sulfate. After electrophoresis, the proteins were transferred to nitrocellulose membranes (100 mA for 1 h 30 min). To control for protein loading and transfer differences, membranes were stained with Ponceau S and images were acquired and analyzed using the 440CF Kodak Imaging System (Kodak, New Haven, CT). The membranes were washed and subsequently blocked in PBS-Tween buffer containing 5.0% skim milk and 0.05% Tween for 2 h at room temperature and subsequently incubated overnight at 4°C with a primary antibody directed against CuZnSOD (SOD-101; Stressgen, Victoria, Canada), MnSOD (SOD-111; Stressgen), CAT (ab16731; Abcam; Cambridge, MA), GPX (ab16798; Abcam or TxnRd1; 07–613; Upstate Cell Signaling Solutions). Primary antibodies were diluted 1:1,000 for CuZnSOD and TxnRd1, whereas MnSOD, catalase, and GPX were diluted 1:2,000. This step was followed by incubation at room temperature with a horseradish peroxidase-antibody conjugate (1:2,000) directed against the primary antibody for 1 h. The membranes were treated with chemiluminescent reagents (ECL Western Blotting Detection Reagent Kit #RPN2106; Amersham Bio) and exposed to light-sensitive film. Images of these films were captured and analyzed using the 440CF Kodak Imaging System (Kodak). Optical density values for 12 h MV are expressed as percentage of normalized control optical densities.

HO-1. We measured HO-1 in our experiments because induction of this enzyme has been associated with enhanced resistance to oxidative stress (37, 39). HO-1, although not a direct scavenger of reactive oxygen species, degrades heme proteins and leads to the formation of biliverdin. Importantly, biliverdin is subsequently reduced to bilirubin on interaction with oxidative species, intracellular DCF can be measured using fluorescent microscopy. In the current experiments we assessed DCF emissions in diaphragm fiber bundles using wide-field fluorescence microscopy. Compared with diaphragms from control animals, DCF emissions were 38% higher in diaphragm bundles from MV animals (P < 0.05; Fig. 1A). This is a clear suggestion of elevated oxidant production in intact diaphragm muscle bundles from mechanically ventilated animals.

RESULTS

Physiological response to MV. Heart rate (HR) and systolic blood pressure (SBP) were maintained within a physiological range during the MV protocol (HR range = 300–420 beats/min; SBP range = 70–130 mmHg). Identical to our previous mechanical ventilation experiments, arterial pH and partial pressures of O2 and CO2 were maintained within a normal range (8, 29, 35, 43). There were no significant differences in body weight between the groups (control = 0.330 g ± 0.01 vs. MV = 0.352 g ± 0.01) before the experimental protocol, and the 12-h experimental protocol did not alter body weight (P < 0.05). This indicates that our hydration and nutrition regimen was adequate. In addition, none of the MV animals tested positive for gram-positive or gram-negative bacteria and there were no visual abnormalities of the lungs or peritoneal cavity, indicating our stringent aseptic surgical technique was successful in preventing infection.

DCFH oxidation. DCFH-DA is capable of passive diffusion through the cell membrane of intact cells, including skeletal muscle fibers (1, 3, 23, 31). Upon entry into the cell, the diacetate is cleaved from DCFH by intracellular esterases, thereby making it impermeable to exit the membrane. After interaction with oxidative species, intracellular DCF can be measured using fluorescent microscopy. In the current experiments we assessed DCF emissions in diaphragm fiber bundles using wide-field fluorescence microscopy. Compared with diaphragms from control animals, DCF emissions were 38% higher in diaphragm bundles from MV animals (P < 0.05; Fig. 1A).

Fig. 1. Effect of 12 h of controlled mechanical ventilation (MV) on dichlorofluorescein (DCFH) oxidation in the diaphragm. A: average diaphragmatic fluorescence for control (CON) and MV groups. Values are expressed as means ± SE. AU, arbitrary units. *Significantly different vs. CON (P < 0.05). B: representative photo of CON diaphragm fiber bundle loaded with DCFH. C: representative photo of MV diaphragm fiber bundle loaded with DCFH.
TAC and total glutathione. Compared with control, a significant \((P < 0.05)\) depletion of diaphragmatic TAC was observed in diaphragm tissue from MV animals (Fig. 2). Additionally, MV resulted in a significant decrease in total glutathione content compared with controls \((P < 0.05)\). Diaphragmatic glutathione levels were 58% of those values obtained from control samples (Fig. 3).

Antioxidant enzyme mRNA. Diaphragmatic mRNA expression levels for antioxidant enzymes assessed by quantitative real-time PCR are reported in Table 1. The diaphragms of MV animals exhibited increased \((P < 0.05)\) mRNA expression for HO-1, MnSOD, and TxnRd1 genes. No change in the mRNA expression of CuZnSOD, CAT, or GPX was detected.

Protein content of antioxidant enzymes. The diaphragmatic antioxidant proteins assessed included HO-1, GPX, CuZnSOD, MnSOD, CAT, and TxnRd1. HO-1 protein levels were higher \((P < 0.05)\) in the MV diaphragms compared with controls (Fig. 4). Moreover, protein levels for GPX and CuZnSOD were \(-1.7-\) and \(-1.3\)-fold lower \((P < 0.05)\), respectively, in the diaphragms of mechanically ventilated animals (Figs. 5B and 6A). In contrast, no significant change in protein levels of MnSOD, CAT, or TxnRd1 was detected (Figs. 5A, 6B, and 7).

DISCUSSION

Overview of principal findings. These are the first experiments to investigate the effects of prolonged mechanical ventilation on diaphragmatic oxidant production and redox buffer capacity. Our results divulge several major findings. Most notably, diaphragms from mechanically ventilated animals exhibited increased intracellular oxidant production and a compromised antioxidant buffering capacity. Our data also reveal that MV promotes changes in gene expression of key antioxidants in the diaphragm at both the mRNA and protein levels. Interestingly, the occurrence of decreased protein levels of select antioxidant proteins was not due to altered levels of mRNA expression. Collectively, these results support the hypothesis that the development of MV-induced diaphragmatic oxidative stress is the consequence of both an elevated intracellular oxidant production in conjunction with a decrease in the antioxidant-buffering capacity. A detailed discussion of these findings and a critique of the experimental model follows.

MV increases intracellular diaphragmatic oxidant production. Our experiments reveal that diaphragms from mechanically ventilated animals exhibit increased levels of intracellular oxidant production compared with control diaphragms. The observation that diaphragmatic inactivity results in increased oxidant production agrees with the report by Lawler et al. (18) indicating that inactive locomotor skeletal muscle (due to hindlimb suspension) also promotes increases in oxidant levels.

Importantly, oxygenation of DCFH has been shown to be a valid and reliable marker of oxidant production in several cell types, including skeletal muscle (1, 23, 24). It is noteworthy that DCFH reacts with numerous oxidants, including \(\text{H}_2\text{O}_2\), \(\cdot \text{OH}\), and \(\text{NOO}^-\). Therefore, the oxidation of DCFH does not reveal the individual source of the oxidant(s) production. Hence the identification of the oxidant-generating pathways involved in the development of oxidative stress in the mechanically ventilated diaphragms merits further investigation.

What are the sources of oxidant production in the diaphragm during prolonged MV? At present, a definitive answer to this question is not available. However, potential oxidant-producing pathways in inactive skeletal muscle include nonphagocytic NADPH oxidase, xanthine oxidase, nitric oxide synthase, and the release of reactive iron. Determining which of these pathways are primary contributors to MV-induced oxidative injury in the diaphragm remains an important area for future research.

TAC is diminished in the diaphragm following MV. The decreased total antioxidant capacity observed in the mechanically ventilated diaphragms was not unexpected because the presence of oxidative stress in these diaphragms has previously been reported (5, 34, 43). Impaired intracellular antioxidant defenses undoubtedly increase the susceptibility of the diaphragm to oxidative damage when oxidant production is increased. Although the measurement of TAC has been used as a general indicator of the total antioxidant status in locomotor skeletal muscle (18, 30), this is the first report to use this technique to evaluate the antioxidant capacity of the diaphragm following MV.

Disturbances in the antioxidant capacity of the diaphragm following MV are also supported by the finding that total glutathione is lowered in diaphragms from MV animals. Indeed, inasmuch as \(-99\%\) of glutathione in the cell exists in the
reduced form (GSH; Ref. 21), measurement of intracellular GSH level is an excellent indicator of total glutathione levels. In mammalian cells, GSH functions as the primary nonenzymatic antioxidant within the cell and, therefore, a decrease in total glutathione is indicative of a depressed antioxidant buffering capacity in the cell (17, 21).

Diaphragmatic antioxidant enzyme expression following MV. Only limited data exist regarding the impact of MV on the activities of key diaphragmatic antioxidants (13, 34). Furthermore, to date, no data exist regarding the protein levels of key antioxidant enzymes in the diaphragm following MV. In the present study, we observed a significant MV-induced decrease in diaphragmatic protein levels of both CuZnSOD and GPX. It seems likely that a decline in diaphragmatic levels of CuZnSOD is physiologically significant because CuZnSOD is the major cytosolic superoxide scavenger (10). With respect to CuZnSOD activity, previous investigations have reported both an increase (34) and decrease (13) in CuZnSOD activity within diaphragms of mechanically ventilated animals. Although the reported decrease in diaphragmatic CuZnSOD activity following MV is consistent with reduced CuZnSOD protein levels, the current results disagree with a previous study from our laboratory indicating that MV results in an increase in diaphragmatic CuZnSOD activity (34). The explanation for this discrepancy is unclear and warrants further study.

It is interesting that the observed decreases in CuZnSOD and GPX protein levels in the present study were not associated with decreased levels of mRNA expression for either protein. Clearly, factors other than the amount of mRNA expression dictate the protein levels of these antioxidant proteins in the diaphragm during MV. In this regard, decreased rates of mRNA translation and/or enhanced degradation of the antioxidant protein could be responsible for dissociation between mRNA and proteins levels in our experiments.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>GenBank mRNA</th>
<th>MV/Con</th>
<th>P Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HO-1</td>
<td>J02772</td>
<td>↑ 23.0</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>CAT</td>
<td>M11670</td>
<td>1.0</td>
<td>NSD</td>
</tr>
<tr>
<td>GPX</td>
<td>M21210; X12367; S41066; BC058438; X07365</td>
<td>1.0</td>
<td>NSD</td>
</tr>
<tr>
<td>MnSOD</td>
<td>Y00497; BC070913</td>
<td>1.6</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
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<td>M21060; Y00404; BC058148; X05634</td>
<td>↓ 1.1</td>
<td>NSD</td>
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<tr>
<td>TxxRd1</td>
<td>AF108213; U63923; AF220760</td>
<td>↑ 2.4</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>β-Glucuronidase</td>
<td>Y00717; M13962</td>
<td>1.0</td>
<td>NSD</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 7/group). MV, controlled mechanical ventilation; Con, control; HO-1, heme oxygenase-1; CAT, catalase; GPX, glutathione peroxidase; MnSOD, manganese superoxide dismutase; CuZnSOD, copper-zinc superoxide dismutase; TxxRd1, thioredoxin reductase-1. *P value calculated by 1-way ANOVA on linear, normalized (β-glucuronidase) input values. NSD, not significantly different.
In mammalian cells, TxnRd1 functions as a hydroperoxide reductase, thus exhibiting important cellular antioxidant properties (44). Interestingly, MV did not alter TxnRd1 protein levels in the diaphragm, although there was a 2.4-fold increase in the mRNA expression of this enzyme. This may be the result of a decreased rate of TxnRd1 protein synthesis or increased rate of TxnRd1 protein degradation. Regardless of the explanation for this finding, the observed redox disturbances in the diaphragm following MV are apparently not associated with alterations in TxnRd1 protein levels.

MnSOD is the principal superoxide scavenger in the mitochondria (42). Although significant increases in the mRNA expression of MnSOD were detected in the diaphragm following MV, no changes in the level of MnSOD protein was observed. However, we previously showed that 12 h of MV resulted in no diaphragmatic contractile activity and because this mode has relevance in several clinical situations. For example, MV is used in adult patients in numerous circumstances (i.e., drug overdose, spinal cord injury, and surgery) and it is also commonly used in pediatric patients (11).

We selected pentobarbital sodium as the general anesthetic in these experiments because of evidence that this anesthetic, when used in low doses, does not negatively impact diaphragm contractile function, does not promote muscle atrophy, and is not associated with oxidative stress in skeletal muscle (5, 19, 35, 43). During our experimental MV protocol, we monitor arterial blood-gas levels (i.e., PO2 and PCO2) and pH to maintain adequate alveolar ventilation and to minimize the occurrence of variations. Additionally, we ensure the prevention of oxidant radiation of heme to produce carbon monoxide, iron, and biliverdin. Although the release of iron from heme may potentially lead to prooxidant effects, the induction of HO-1 is generally thought to convey protection against oxidative damage (32). For example, biliverdin possesses antioxidant properties (39) and can be converted into bilirubin; which exhibits potent antioxidant characteristics (37). Indeed, bilirubin has been shown to protect cells in vitro from hydrogen peroxide concentrations 10,000-fold above physiological levels (2). Nonetheless, it is unclear as to whether the observed increase in HO-1 levels in the diaphragms of MV animals acted as a prooxidant or an antioxidant. This is an important topic for future work.

Critique of the experimental model. Given the invasive nature of removing diaphragm samples, animal models must be used to study the effects of MV on respiratory muscle biochemistry. Considerations in the choice of an animal model include both practical considerations (i.e., size of the animal) and the applicability of the animal model to humans. In this regard, we chose the rat model for several reasons. First, adult rats are of adequate size to permit surgical procedures and removal of several arterial blood samples for blood gas analysis during prolonged MV. Furthermore, and most importantly, human and rat diaphragms are similar in fiber-type composition, gross anatomical features, and function (22, 27). Additionally, we chose to use controlled MV because this mode results in no diaphragmatic contractile activity and because this mode has relevance in several clinical situations. We selected pentobarbital sodium as the general anesthetic in these experiments because of evidence that this anesthetic, when used in low doses, does not negatively impact diaphragm contractile function, does not promote muscle atrophy, and is not associated with oxidative stress in skeletal muscle (5, 19, 35, 43). During our experimental MV protocol, we monitor arterial blood-gas levels (i.e., PO2 and PCO2) and pH to maintain adequate alveolar ventilation and to minimize the occurrence of variations. Additionally, we ensure the prevention of oxidant

Fig. 6. Effect of 12 h of controlled mechanical ventilation on diaphragmatic levels of copper-zinc superoxide dismutase (CuZnSOD; A) and manganese superoxide dismutase (MnSOD; B) protein. Optical density values are expressed as % of control ± SE. *Significantly different vs. CON (P < 0.05).

Fig. 7. Effect of 12 h of controlled mechanical ventilation on diaphragmatic thioredoxin reductase-1 (TxnRd1) protein levels. Optical density values are expressed as % of control ± SE. *Significantly different vs. CON (P < 0.05).
generation from excessive $P_O_2$ tension due to the administration of hyperoxic gas. Moreover, we did not employ an anesthetized and spontaneously breathing control group in the current experiments because we demonstrated that prolonged exposure to pentobarbital sodium in spontaneously breathing rats does not promote oxidative stress in the diaphragm (35, 43).

To detect oxidant production in bundles of intact diaphragm fibers, we measured the fluorescence of the highly sensitive redox probe, DCFH, in diaphragm fiber bundles using wide-field fluorescence microscopy. This technique has been shown to be a robust measure of intracellular oxidant production in intact diaphragm fiber bundles (1, 23, 31, 46). Importantly, this technique does not suffer from many of the limitations inherent to techniques employed to measure intracellular oxidant production that require disruption of the muscle fibers. Indeed, the maintenance of cellular compartmentalization is particularly important when attempting to extrapolate in vitro measurement of tissue oxidant production to in vivo conditions. DCFH-DA is a cell-permeable dye capable of diffusing into the outermost surface fibers of the diaphragm (1, 23, 31). Although the bundles of diaphragm fibers used in our DCFH experiments were not perfused, it is unlikely that the superficial muscle fibers imaged were hypoxic due to a diffusion limitation of oxygen into fibers. Furthermore, the $O_2$ tension measured in our organ bath (~600 Torr) surrounding the muscle samples does not significantly affect DCF fluorescence compared with organ baths containing a lower $O_2$ tension (1).

Note that we chose to incubate fiber bundles at 25°C because the diaphragm fibers are more physiologically stable at this temperature compared with incubations at higher temperatures (i.e., 37°C) where leakage of the probe may occur (23). However, because our muscle samples were incubated at 25°C rather than 37°C, it is possible that our experimental approach may actually underestimate the degree of oxidant production in the diaphragm fiber bundles (1, 23).

In summary, these experiments provide new and important information regarding the impact of MV on diaphragmatic oxidant production and redox status within the diaphragm. Our results reveal that as few as 12 h of MV result in increased oxidant production in diaphragm muscle fibers. Furthermore, MV results in altered diaphragmatic antioxidant gene expression and depression of the antioxidant capacity of these muscle fibers. Collectively, these results are consistent with the hypothesis that diaphragmatic inactivity during MV promotes oxidative stress in the diaphragm due to both increased oxidant production and a decrease in cellular antioxidant capacity. Hence, these novel findings provide the foundation for future experiments that will lead to specific therapeutic modalities to retard MV-induced oxidative stress in the diaphragm; prevention of MV-induced oxidative injury in the diaphragm could be helpful in reducing the incidence of problems in weaning patients from mechanical ventilation.

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