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Mechanical ventilation-induced oxidative stress in the diaphragm

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Abstract

Prolonged mechanical ventilation (MV) results in oxidative damage in the diaphragm; however, it is unclear whether this MV-induced oxidative injury occurs rapidly or develops slowly over time. Furthermore, it is unknown whether both soluble (cytosolic) and insoluble (myofibrillar) proteins are equally susceptible to oxidation during MV. These experiments tested two hypotheses: 1) MV-induced oxidative injury in the diaphragm occurs within the first 6 h after the initiation of MV; and 2) MV is associ-ated with oxidative modification of both soluble and insoluble proteins. Adult Sprague-Dawley rats were randomly divided into one of seven experimental groups: 1) control (n =8); 2) 3-h MV (n = 8); 3) 6-h MV (n = 6); 4) 18-h MV (n = 8); 5) 3-h anesthesia-spontaneous breathing (n = 8); 6) 6-h anesthesia-spontaneous breathing (n = 6); and 7) 18-h anesthesia-spontaneous breathing (n ⊠ 8). Markers of oxidative injury in the diaphragm included the measurement of reactive (pro-tein) carbonyl derivatives (RCD) and total lipid hydroperox-ides. Three hours of MV did not result in oxidative injury in the diaphragm. In contrast, both 6 and 18 h of MV promoted oxidative injury in the diaphragm, as indicated by increases in both protein RCD and lipid hydroperoxides. Electro-phoretic separation of soluble and insoluble proteins indi-cated that the MV-induced accumulation of RCD was limited to insoluble proteins with molecular masses of ⊠200, 120, 80, and 40 kDa. We conclude that MV results in a rapid onset of oxidative injury in the diaphragm and that insoluble proteins are primary targets of MV-induced protein oxidation.

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Prolonged mechanical ventilation (MV) results in oxidative damage in the diaphragm; however, it is unclear whether this MV-induced oxidative injury occurs rapidly or develops slowly over time. Furthermore, it is unknown whether both soluble (cytosolic) and insoluble (myofibrillar) proteins are equally susceptible to oxidation during MV. These experiments tested two hypotheses: *1)* MV-induced oxidative injury in the diaphragm occurs within the first 6 h after the initiation of MV; and *2)* MV is associated with oxidative modification of both soluble and insoluble proteins. Adult Sprague-Dawley rats were randomly divided into one of seven experimental groups: *1)* control ($n = 8$); *2)* 3-h MV ($n = 8$); *3)* 6-h MV ($n = 6$); *4)* 18-h MV ($n = 8$); *5)* 3-h anesthesia-spontaneous breathing ($n = 8$); *6)* 6-h anesthesia-spontaneous breathing ($n = 6$); and *7)* 18-h anesthesia-spontaneous breathing ($n = 8$). Markers of oxidative injury in the diaphragm included the measurement of reactive (protein) carbonyl derivatives (RCD) and total lipid hydroperoxides. Three hours of MV did not result in oxidative injury in the diaphragm. In contrast, both 6 and 18 h of MV promoted oxidative injury in the diaphragm, as indicated by increases in both protein RCD and lipid hydroperoxides. Electrophoretic separation of soluble and insoluble proteins indicated that the MV-induced accumulation of RCD was limited to insoluble proteins with molecular masses of ~200, 120, 80, and 40 kDa. We conclude that MV results in a rapid onset of oxidative injury in the diaphragm and that insoluble proteins are primary targets of MV-induced protein oxidation.

respiratory muscles; muscle atrophy; free radicals; protein oxidation; weaning from mechanical ventilation; muscle injury

MECHANICAL VENTILATION (MV) is used to maintain ventilation in patients who are incapable of maintaining adequate alveolar ventilation on their own. Respiratory failure, neuromuscular diseases, drug overdoses, and recovery from general anesthetics are common indications for the use of MV (9). Problems in weaning patients after prolonged MV have been reported in a large number of individuals, and it is postulated that a common cause of difficult weaning is diaphragmatic force and endurance deficits (2, 11, 31–33). This notion is strongly supported by several recent animal studies demonstrating that controlled MV is associated with significant diaphragmatic atrophy and contractile dysfunction (1, 13, 19, 22, 27–29).

Our laboratory has recently discovered that 18 h of controlled MV results in an increase in oxidized proteins in the diaphragm, as indicated by elevated protein carbonyls (29). In the context of MV-induced diaphragmatic atrophy, an increase in protein oxidation is important, because moderately oxidized proteins are sensitive to proteolytic attack by the proteasome pathway of protein degradation (reviewed in Refs. 4 and 30). Therefore, oxidative modification of proteins could accelerate protein degradation in the diaphragm, resulting in diaphragmatic atrophy during prolonged MV.

Of further interest is the possibility that the oxidative modification of diaphragm proteins contributes to the MV-induced diaphragmatic force deficit. Indeed, it is well established that oxidative stress in skeletal muscle can modify several proteins associated with excitation-contraction coupling and contribute to a reduction in muscle force production (reviewed in Refs. 17, 24–26). Therefore, oxidative injury in the diaphragm could have important implications in the etiology of both MV-induced atrophy and contractile deficits in the diaphragm.

At present, numerous questions associated with MV-induced diaphragmatic oxidative injury remain unanswered. For example, it is unknown whether MV-induced oxidative injury occurs within the first 6 h of MV or develops slowly over time. Furthermore, it is unclear which diaphragmatic proteins are oxidatively modified during MV. Therefore, the objectives of this experiment were twofold: *1)* to determine whether oxidative modification of diaphragmatic proteins occurs rapidly after the initiation of MV; and *2)* to establish whether both soluble and insoluble proteins are oxidatively

modified during MV. On the basis of evidence that immobilization of locomotor skeletal muscles results in progressive oxidative injury in muscle fibers (12), we hypothesized that MV-induced oxidative injury in the diaphragm would develop gradually within the first 6 h of MV. Furthermore, we hypothesized that MV-induced oxidative damage in the diaphragm would target both soluble and insoluble proteins in skeletal muscle. This postulate evolved from a report indicating a differential susceptibility of soluble and insoluble skeletal muscle proteins to free radical damage (7).

METHODS

Experimental Animals

These experiments were approved by the University of Florida animal use committee and followed the guidelines for animal experiments established by the American Physiological Society. Healthy, young adult (female, 4-mo-old) Sprague-Dawley rats were individually housed and fed rat chow and water ad libitum and were maintained on a 12:12-h light-dark cycle for 3 wk before initiation of these experiments.

Experimental Design

To examine the time course of MV-induced contractile dysfunction in the diaphragm, animals were randomly assigned to one of seven experimental groups (Fig. 1).

Protocol for control animals. The animals in the control group were free of intervention before measurement of diaphragmatic biochemical properties. That is, these animals were not mechanically ventilated or exposed to long-term anesthesia before study. Animals in the control group received an intraperitoneal (IP) injection of pentobarbital sodium (50 mg/kg body wt). After a surgical plane of anesthesia was achieved, the diaphragm was rapidly removed, and segments from the ventral costal region were rapidly frozen in liquid nitrogen and stored at -80°C for subsequent analysis of oxidant damage.

Protocol for anesthetized, spontaneously breathing animals. To determine whether long-term anesthesia (i.e., pentobarbital sodium) impairs diaphragmatic contractile properties, our experimental design included three groups of anesthetized, spontaneously breathing (SB) animals. Selected diaphragmatic biochemical properties were studied after 3, 6, or 18 h of SB. Animals were anesthetized with an IP injection of pentobarbital sodium (50 mg/kg), and, after a surgical plan of anesthesia was reached, a venous catheter was inserted in the jugular vein to permit the infusion of isotonic saline and pentobarbital sodium when necessary.

Animals were then tracheostomized, and a surgical plane of anesthesia was maintained throughout the experimental period by constant infusion of pentobarbital sodium ($\sim$10 mg$\cdot$kg$^{-1}\cdot$h^{-1}). An arterial catheter was placed in the carotid artery, and arterial blood samples ($\sim$100 µl/sample) were removed during the first and last hour from each experimental group. Arterial blood samples were analyzed for arterial P_{CO_2} (Pa_{CO_2}), P_{O_2} (Pa_{CO_2}), and pH by using a blood-gas analyzer (Instrumentation Laboratories, model 1610). Before analysis, the analyzer was calibrated by using standardized gases and pH solutions. To avoid infection, strict aseptic techniques were used during all invasive procedures.

Body temperature was maintained at $\sim$37°C by use of a recirculating heating blanket. Animals in the SB groups were constantly monitored by a technician and received continuing care. Continuing care during anesthesia of the experimental groups included expressing the bladder, removing upper airway mucus, lubricating the eyes, rotating the animal, and passive movement of the limbs. This care was maintained throughout the experimental period at 1-h intervals. Furthermore, both heart rate and blood pressure were measured and recorded at 1-h intervals throughout the experiment. Finally, to reduce airway secretions, animals in the SB groups received intramuscular injections of glycopyrrolate (0.04 mg/kg) every 2 h.

Throughout the period of SB, animals received enteral nutrition by use of the American Institute of Nutrition-76 rodent diet with a nutrient composition of 15% proteins, 35% lipids, 50% carbohydrates, and vitamins and minerals (Research Diets, Brunswick, NY). Our planned feeding schedule was designed to provide an isocaloric diet with the nutrients administered every 2 h via a gastric tube; the total daily administration of 69 ml is equivalent to 69 kcal/day. Body fluid homeostasis was maintained via the administration of 2.0-ml$\cdot$kg$^{-1}\cdot$h^{-1} intravenous electrolyte solution. On completion of the prescribed time period of SB (i.e., 3, 6, or 18 h), segments of the costal diaphragm were removed and rapidly frozen in liquid nitrogen and stored at -80°C for subsequent analysis of oxidant damage.

Protocol for mechanically ventilated animals. All surgical procedures were performed by using the aseptic technique. Animals randomly selected for MV were anesthetized with an IP injection of pentobarbital sodium (50 mg/kg body wt). After reaching a surgical plane of anesthesia, the animals were tracheostomized and mechanically ventilated by using a volume-driven, small-animal ventilator (Harvard Apparatus, Cambridge, MA). The ventilator delivered all breaths; hence, this mode of ventilation (i.e., controlled MV) results in complete diaphragmatic inactivity. The tidal volume was established at $\sim$1 ml/100 g body wt, with a respiratory rate of 80 breaths/min. This respiratory rate was selected to mimic
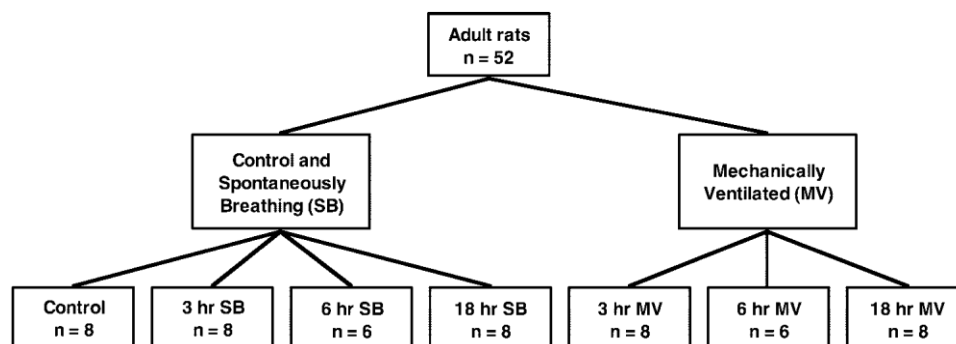

Fig. 1. Schematic illustration of the experimental design used in these experiments. SB, spontaneously breathing; MV, mechanically ventilated.

the breathing frequency of adult rats at rest. Additionally, positive end-expiratory pressure (PEEP) of 1 cmH$_2$O was used throughout the protocol.

An arterial catheter was placed in the carotid artery for constant measurement of blood pressure and periodic blood sampling for analysis of arterial pH and blood gases. Arterial blood samples (~100 μl/sample) were removed during the first and last hour of MV and analyzed for Pa$_{CO_2}$, Pa$_{O_2}$, and arterial pH by using a blood-gas analyzer (Instrumentation Laboratories, model 1610). Before analysis, the analyzer was calibrated by using standardized gases and pH solutions.

A venous catheter was inserted in the jugular vein to permit the infusion of isotonic saline and pentobarbital sodium when necessary. Anesthesia was maintained over the entire period of MV by continuous infusion of pentobarbital sodium (~10 mg/kg body wt). Note that we continuously monitored the level of anesthesia in our animals by several methods (i.e., monitoring heart rate, blood pressure, and corneal-lid reflexes). Body temperature was maintained at ~37°C by use of a recirculating heating blanket. Additionally, heart rate and electrical activity of the heart were monitored via a lead II ECG by using needle electrodes placed subcutaneously. Identical to the SB experiments, strict aseptic techniques were used during all invasive procedures.

Throughout MV, animals received enteral nutrition by use of the aforementioned American Institute of Nutrition-76 rodent diet. This planned feeding schedule was identical to the routine described for the SB animals. Furthermore, body fluid homeostasis was maintained via the administration of 2.0 ml·kg^{-1}·h^{-1} intravenous electrolyte solution.

Continuing care during MV included expressing the bladder, removing airway mucus, lubricating the eyes, rotating the animal, and passive movement of the limbs. This care was maintained throughout the experimental period at hourly intervals. Finally, identical to the protocol used in the SB animals, repeated intramuscular injections of glycopyrrolate (0.04 mg·kg^{-1}·2 h^{-1}) were employed to reduce airway secretions. On completion of MV, segments of the costal diaphragm were removed, rapidly frozen in liquid nitrogen, and stored at -80°C for subsequent analysis of oxidant damage.

Biochemical Measurements

Measurement of reactive carbonyl derivatives. Oxidative modification of proteins by free radical species and other reactive species results in the formation of carbonyl groups into amino acid side chains by a site-specific mechanism. These carbonyl groups or reactive carbonyl derivatives (RCD) can be identified by using sensitive assays. The RCD in diaphragms from all experimental groups were determined by both spectrophotometry and immunoblot methods. The spectrophotometric assay was used to determine total RCD in diaphragm. Briefly, a section (25–45 mg) of the costal diaphragm was homogenized and assayed to quantitatively determine the levels of RCD in control, SB, and MV animals, as described by Reznick and Packer (26).

To determine the molecular weights of oxidized proteins in the diaphragm, soluble and insoluble proteins were separated by centrifugation (11,000 *g* at 4°C for 10 min). Protein (2 μg) from the soluble and insoluble fractions were then individually separated via polyacrylamide gel electrophoresis; RCD were then measured by Western blot by using a protocol recommended by the manufacturer (Intergen, Oxy-Blot protein oxidation detection kit, S7150, Purchase, NY). A brief overview of this protocol follows.

The carbonyl groups in the protein side chains of both soluble and insoluble proteins were derivatized to 2,4-dinitrophyenylhydrazone (DNP). These DNP-derivatized protein samples were separated in duplicate by using polyacrylamide gel electrophoresis via 4–20% gradient polyacrylamide gels containing 0.1% SDS. After electrophoresis, the proteins were transferred to nitrocellulose membranes. The membranes were then soaked in PBS buffer containing 3% skim milk, 0.05% Tween, and 0.05% sodium azide and subsequently incubated with a primary antibody, specific to the DNP moiety of the proteins. This step was followed by incubation with a horseradish peroxidase antibody conjugate directed against the primary antibody. The membranes were then treated with chemiluminescent reagents (luminol and enhancer) and exposed to light-sensitive film. Images of these films were captured and analyzed by using computerized image analysis (Gel Doc 2000, BioRad, Hercules, CA). The oxidative status of different molecular weight proteins was determined by comparing the signal intensity of the same protein in different lanes of the various gels.

Furthermore, to determine the identity of selected oxidized proteins, membranes containing diaphragmatic insoluble proteins were stripped of the DNP antibody and were then reprobed with monoclonal antibodies specific for rat skeletal muscle actin and myosin heavy chains. More specifically, to determine whether the 40- and 200-kDa bands were actin and myosin, membranes were sequentially stripped and reprobed with actin monoclonal (anti-a-sarcomeric actin, clone 5c5, no. A2172, Sigma Chemical, St. Louis, MO) and myosin monoclonal [anti-myosin heavy chain (all adult isoforms), clone A4.1025, DSHB, University of Iowa, Iowa City, IA] antibodies. Importantly, our laboratory has confirmed the specific reactivity of these monoclonal antibodies with the respective purified proteins. Membranes were incubated at 37°C for 30 min with membrane-stripping buffer (Restore Western Blot Stripping Buffer, no. 21059, Pierce, Rockford, IL) followed by 1 h of blocking in PBS buffer containing 3% skim milk and 0.05% Tween. Myosin and actin antibodies were applied to membranes at concentrations of 1:500 and 1:1,000 in blocking buffer, respectively, and incubated for 1 h. Membranes were rinsed twice and then washed with PBS buffer containing 0.05% Tween for 30 min before and after the secondary antibody incubation. The secondary antibody (anti-mouse, HRP conjugated) was diluted 1:2,000 in PBS containing 0.05% Tween and applied to the membrane for 1 h. The membranes were treated with chemiluminescent reagents and exposed to light-sensitive film, as described above.

Measurement of total lipid hydroperoxides. Assessment of lipid peroxidation in tissues can be estimated by measurement of tissue levels of total hydroperoxides. Tissue hydroperoxides were determined by using the modifications of the ferrous oxidation/xylenol orange technique described by Hermes-Lima et al. (8). Details of these techniques are contained in previous reports from our laboratory (3, 17). In our hands, the within-day coefficient of variation for this assay is low (~5%), but the between-day coefficient of variation is considerably larger (~12%). Hence, because of experimental timing and the large number of samples to be assayed, by necessity, these assays were performed on different days. To reduce the between-day variability in these measurements, an equal number of diaphragm samples from control, SB, and MV samples was assayed on each day, and the total lipid hydroperoxide concentrations in both SB and MV diaphragms were then normalized as a percentage of control.

Table 1. *Animal body weights before and after the experimental treatments (i.e., spontaneous breathing or mechanical ventilation*

Experimental Group	Before Treatment	After Treatment
Control	296±5	
SB-3 h	283±7	284±7
MV-3 h	279±6	282±6
SB-6 h	297±12	295±11
MV-6 h	277±6	276±5
SB-18 h	275±11	266±10
MV-18 h	296±15	293±14

Values are means ± SD in g. SB, spontaneous breathing; MV, mechanical ventilation. No differences existed between the experimental groups before or after the experimental treatment.

Statistical Analysis

Comparisons between groups for each dependent variable measured were made by a two-way ANOVA. Significance was established at $P < 0.05$. Where significant differences existed, a Scheffé test was used post hoc.

RESULTS

Systemic and Biological Response to MV

One animal that completed the prescribed period of MV was eliminated from our analysis because postmortem analysis revealed evidence of lung barotrauma and significant disturbance of blood-gas homeostasis. No animals were eliminated due to infection. Of the animals included in our analysis, initial and final body weights did not differ ($P > 0.05$) between the experimental groups. Note that 3–18 h of MV did not significantly alter ($P > 0.05$) animal body weights (Table 1). These results confirm that our program of enteral nutrition and rehydration was adequate.

To determine whether our MV protocol was successful in maintaining homeostasis, we measured arterial blood pressure, pH, and Pa_{CO_2} and Pa_{O_2} in all animals within the first hour of MV and during the final hour of MV. Our data indicate that adequate arterial blood pressure and blood gas/pH homeostasis were maintained during MV. For example, Pa_{O_2} and Pa_{CO_2} during the last hour of MV in the 18-h MV animals ranged from 71–82 and 32–36 Torr, respectively. Systolic blood pressure and arterial pH ranged from 95 to 115 mmHg and 7.42 to 7.45, respectively. Similar values were recorded in both the 3- and 6-h MV groups.

In contrast to the MV animals, measurements of arterial blood-gas tensions and pH in the SB animals revealed that these animals experienced hypoxemia, hypercapnia, and mild acidosis during the experimental period. For example, Pa_{O_2} and Pa_{CO_2} ranged from 45 to 65 and 49 to 68 Torr, respectively, during the last hour of SB in the 3-, 6-, and 18-h SB animals. These disturbances in blood-gas homeostasis during anesthesia were likely due to hypoventilation resulting from depressed ventilatory drive.

Because sepsis is associated with diaphragmatic contractile dysfunction, strict aseptic techniques were followed throughout the experiments. Our results indi-

cate that the animals included in our analysis did not develop infections during MV or SB. This statement is supported by the observation that microscopic examination of blood revealed no detectable bacteria, and postmortem examination of the lungs (visual) and peritoneal cavity (visual) displayed no detectable abnormalities. Furthermore, both our MV and SB animals were afebrile during the investigation. Finally, during the course of MV and SB, no significant ($P > 0.05$) changes occurred in the body weights of our animals. Collectively, these results indicate that the MV and SB animals used in our analysis were free of significant infection.

Measurements of Diaphragmatic Oxidative Injury

RCD. RCD were measured as a marker of protein oxidation in diaphragms from all experimental groups. Mean (±SE) levels of total RCD (nmol of RCD per mg of protein) in the costal diaphragm from all experimental groups are contained in Fig. 2. No significant differences existed in total RCD (i.e., soluble and insoluble proteins) between control animals and the SB groups. However, compared with control and SB, only 6 and 18 h of MV resulted in significant increases in the levels of total RCD in the diaphragm. Although RCD levels in diaphragms from 18-h MV animals tended to be greater than those from 6-h MV animals, these differences were not significant.

Figure 3 contains two representative Western blots illustrating the RCD content in the costal diaphragm in a control animal vs. animals exposed to MV or SB. Computerized image analysis of these blots revealed that, compared with control and 18 h of SB, 18 h of MV resulted in significant increases in RCD in diaphragmatic insoluble proteins with molecular masses of ~200, 128, 85, and 40 kDa (Fig. 4). Similarly, compared with both control and 6 h of SB, 6 h of MV resulted in an increase in RCD in diaphragmatic insoluble proteins with molecular masses of ~200, 128, and
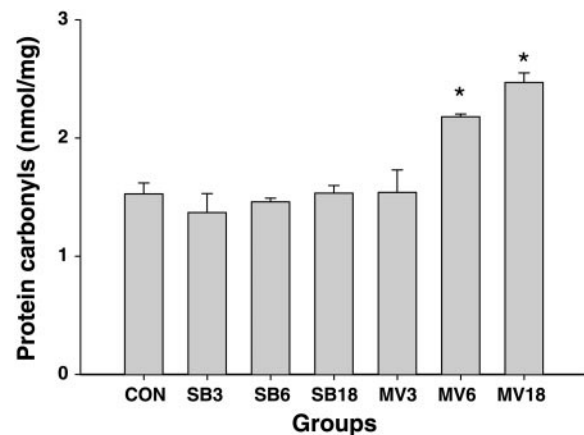

Fig. 2. Spectrophotometric analysis of the accumulation of total reactive carbonyl derivatives (RCD) in diaphragmatic insoluble proteins in all 7 experimental groups: *1*) control (Con); *2*) 3 h of SB (SB3); *3*) 6 h of SB (SB6); *4*) 18 h of SB (SB18); *5*) 3 h of MV (MV3); *6*) 6 h of MV (MV6); and *7*) 18 h of MV (MV18). Values are means ± SE. *Significantly different ($P < 0.05$) from Con, SB6, and SB18.
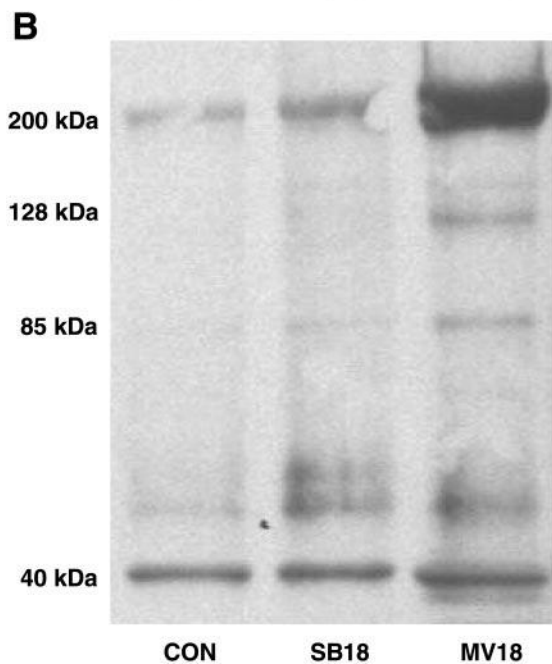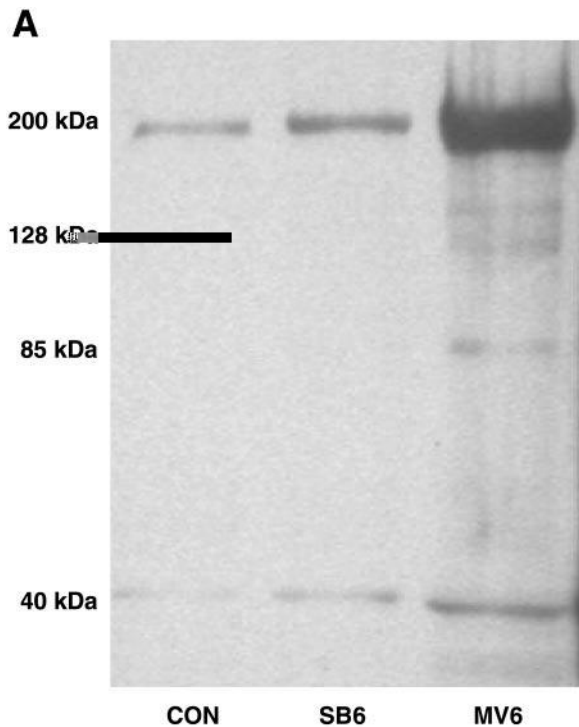
Fig. 3. Representative Western blots illustrating the level of RCD in diaphragmatic insoluble proteins with molecular masses ranging between 200 and 40 kDa. *A*: levels of RCD in diaphragmatic insoluble proteins in Con, SB6, and MV6. *B*: level of RCD in diaphragmatic insoluble proteins in Con, SB18, and MV18.

Fig. 4. Analysis of Western blots of the accumulation of RCD within insoluble proteins in the diaphragm of Con, SB, and MV animals. Values are means ± SE of the optical densities. *Significantly different ($P < 0.05$) from Con, SB6, and SB18. #Significantly different ($P < 0.05$) from MV6.

blots were stripped of the DNP antibody and were then sequentially reprobed for rat skeletal muscle actin and myosin heavy chains (all isoforms). These experiments revealed that the oxidized 200- and 40-kDa proteins were the contractile proteins myosin heavy chain and actin, respectively (Fig. 5).

Lipid hydroperoxides. Tissue levels of lipid hydroperoxides were assessed as markers of lipid peroxidation

85 kDa. Also, compared with 6 h of MV, 18 h of MV resulted in significantly higher RCD in insoluble diaphragmatic proteins with molecular masses of ~200, 128, and 40 kDa. Finally, no significant differences existed in RCD levels of soluble proteins at any molecular mass after MV (data not shown).

To determine the identity of the oxidized insoluble proteins in the diaphragm, representative Western
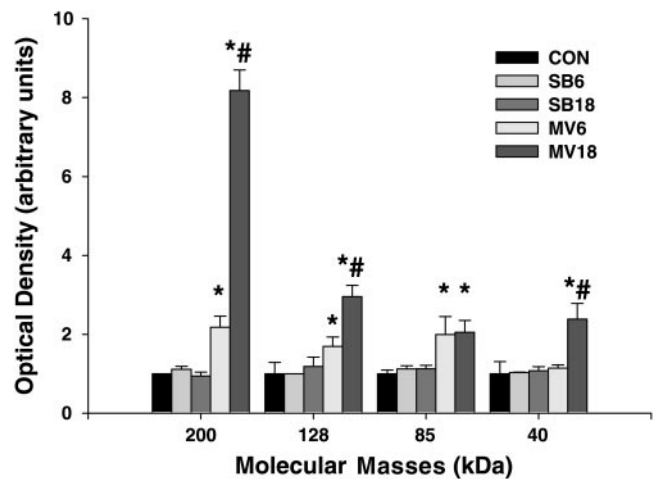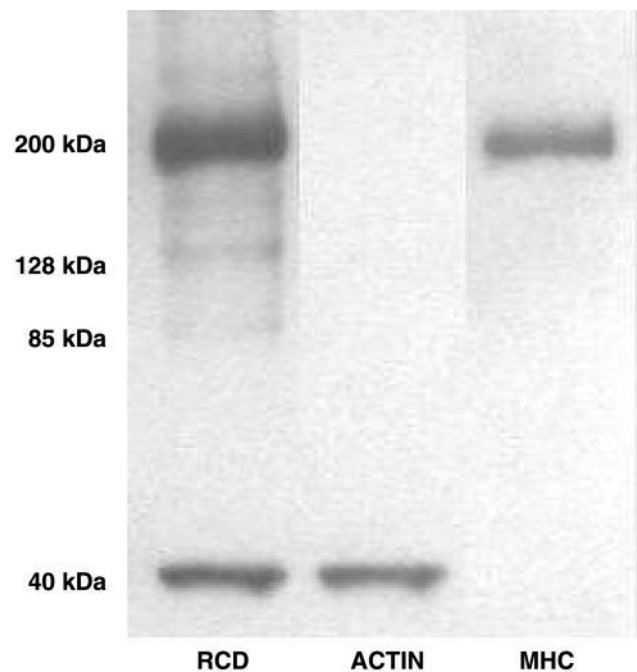

Fig. 5. Illustration of Western blots using monoclonal antibodies to identify oxidized proteins with molecular masses of ~200 to 40 kDa. *Left lane:* RCD in insoluble proteins isolated from the diaphragm of an animal exposed to MV18. *Middle* and *right lanes:* the same membrane stripped of the 2,4-dinitrophyenylhydrazone antibody and then sequentially reprobed with monoclonal antibodies specific for rat skeletal muscle actin and all myosin heavy chain (MHC) isoforms.

in the costal diaphragm. In agreement with the RCD data, no differences in diaphragmatic levels of lipid hydroperoxides existed between control and SB animals. Furthermore, no differences existed in diaphragmatic lipid hydroperoxide levels between control animals and animals exposed to 3 h of MV (Table 2). In contrast, both 6 and 18 h of MV were associated with significant increases in diaphragmatic levels of lipid hydroperoxides (Table 2). Whereas diaphragmatic levels of lipid hydroperoxides tended to be greater in 18-h MV animals compared with 6-h MV animals, these differences did not reach statistical significance.

DISCUSSION

Overview of Principal Findings

These experiments provide new and important information regarding both the time course of MV-induced oxidative injury in the diaphragm and unique details about the insoluble proteins that are preferentially oxidized. Our results reveal that MV-induced oxidative injury in the diaphragm does not occur rapidly after the onset of MV (i.e., <3 h of MV), but is present after 6 h of MV. Furthermore, our data indicate that insoluble proteins (molecular masses of ~200, 128, 85, and 40 kDa) are differentially oxidized during controlled MV. The oxidized proteins with molecular masses of 40 and 200 kDa were identified as being actin and myosin heavy chain, respectively. Collectively, these data support the hypothesis that the onset of MV-induced diaphragmatic oxidative injury occurs within 6 h after the onset of MV and that insoluble proteins are targets for oxidative modification. The biological and clinical significance of these results are discussed in the following sections.

Biological Significance of MV-induced Oxidative Injury

It is well established that radicals and other reactive oxygen species (ROS) are produced in both contracting and inactive skeletal muscles (12, 24). When ROS production in skeletal muscle is high enough to overwhelm biological antioxidant defense systems, oxidative stress occurs. In this regard, oxidants can alter the structure and function of lipids, proteins, and nucleic acids, leading to cellular injury and death (20).

Oxidative injury in skeletal muscle is biologically important because oxidant stress can contribute to both muscle atrophy and contractile dysfunction (reviewed in Refs. 18, 23–25). In the context of MV-induced diaphragmatic atrophy, protein oxidation is significant because oxidized proteins are sensitive to proteolytic attack by proteases (4, 30). Hence, it is possible that oxidative stress plays an important role in MV-induced atrophy of the diaphragm.

Oxidative stress in skeletal muscles is also associated with contractile dysfunction. Indeed, numerous investigators have demonstrated that oxidant stress results in depressed muscle force production at both submaximal and maximal stimulation frequencies (26). Although oxidant injury can impair the function of several skeletal muscle proteins associated with excitation-contraction coupling, the specific mechanism(s) responsible for the oxidant-induced force deficit in muscle is unclear and continues to be an active area of research.

MV-induced Oxidative Injury: Sources of ROS

Historically, it was believed that ROS production is relatively high in contracting skeletal muscle and low in noncontracting muscle. However, extensive work by Kondo et al. (12) has demonstrated that oxidative injury occurs in immobilized locomotor muscle, and this injury contributes to muscle atrophy. Similarly, the present experiments demonstrate that controlled MV leading to diaphragmatic inactivity results in oxidative injury in the diaphragm (Figs. 2 and 4; Table 2). Importantly, our experiments demonstrate that the oxidative damage in the diaphragm associated with MV is not due to the anesthetic, because protein oxidation does not occur in diaphragms of anesthetized, SB animals (Fig. 2).

Although our results clearly indicate that MV is associated with diaphragmatic oxidative injury, these experiments do not reveal the source(s) of ROS production. Pathways capable of producing ROS in skeletal muscle are numerous, and the pathways responsible for production of these oxidants are state dependent (reviewed in Ref. 10). For example, it is possible that the diaphragmatic oxidant production associated with MV could have occurred due to one or more of the following pathways: *1*) generation of superoxide radicals by xanthine oxidase; *2*) nitric oxide produced by nitric oxide synthase; *3*) mitochondrial production of ROS; *4*) ROS created by prostanoid metabolism in conjunction with increased phospholipase A_2 activity; *5*) radical formation due to disruption of iron-containing proteins; *6*) radical generation by invading phagocytic cells; and *7*) NADPH oxidase. Kondo et al. (12) demonstrated that the oxidant production associated with immobilization of locomotor muscles was due, at least in part, to both the xanthine oxidase pathway and reactions involving reactive iron. Nonetheless, at present, the oxidant-producing pathways that are responsible for MV-induced diaphragmatic oxidative in-

Table 2. *Effects of MV on diaphragmatic levels of total lipid hydroperoxides*

	3-h SB	3-h MV	6-h SB	6-h MV	18-h SB	18-h MV
Total lipid hydroperoxides (%control)	0.4±6.5	-10.7±5.8	-3.8±4.8	58.6±8.1*	4.1±5.7	71.8±4.8*

Values are means ± SE expressed as a percentage above or below the values of control animals (nonventilated animals). *Significantly different from control animals at $P < 0.05$.

jury are unknown. This is an important area for future research.

Time Course of MV-induced Oxidative Injury

A new and important finding in this experiment was that MV-induced oxidative injury in the rat diaphragm occurs within 6 h after the initiation of MV. Based on the present data alone, it is unclear whether these findings can be extrapolated to humans. However, if a similar time course of MV-induced diaphragmatic oxidative injury exists in humans, our results would have important clinical implications that are applicable to numerous surgical procedures. Given the potential clinical significance of MV-induced diaphragmatic injury, this is an important area for future research.

Although the present experiment is the first to examine the time course of oxidative injury due to MV-induced inactivity in the diaphragm, previous experiments investigating locomotor skeletal muscle indicate that 8 days of immobilization are required to promote oxidative injury in the rat soleus muscle (12). Whereas both of these experimental models demonstrate that muscular inactivity is associated with oxidative stress, it is important to note that there are significant differences between these two experimental paradigms (i.e., immobilization vs. MV). Indeed, during controlled MV, both the right and left hemidiaphragm are not contracting (19) but are undergoing repetitive passive shortening during mechanical expansion of the lungs (5). In contrast, immobilization of the soleus muscle does not permit muscle shortening. At present, it is unknown whether the rate and types of oxidant production are similar in immobilized muscles compared with mechanically inactive and passively shortened muscles. This is an important topic that warrants further study.

Differential Protein Oxidation in the Diaphragm During MV

Our results indicate that MV-induced protein oxidation in the diaphragm occurs in insoluble proteins. In contrast, no evidence of protein oxidation was found in soluble proteins within the diaphragm. There are at least three explanations for this differential response in MV-induced protein oxidation. First, it is possible that, compared with soluble proteins, the cellular site(s) of ROS formation favored the oxidation of insoluble proteins. Second, it has been postulated that the antioxidant capacity is greater in the cytosol compared with other regions of the cell (21). In this case, compared with other cellular areas (e.g., mitochondria), the cytosol would be better protected against oxidative stress. Finally, it is possible that cytosolic proteins were oxidized during MV, but these damaged proteins were rapidly degraded by proteases. Therefore, if these oxidized proteins were degraded quickly after their formation, they would go undetected in the analysis of RCD. This notion has received support from Radak et al. (21). These investigators hypothesized that the ability of the cytosol to remove oxidatively modified proteins is greater than that of other portions of the cell, because the cytosol hosts the proteasome complex, which is the primary protease that catabolizes oxidatively modified proteins (4, 30).

In regard to oxidative damage to specific insoluble proteins, our results reveal that 18 h of MV resulted in significant oxidation of insoluble proteins at molecular masses of ~200, 128, 85, and 40 kDa, as indicated by increases in the levels of RCD. Although one-dimensional electrophoresis alone does not permit the identification of individual proteins, recognition of the molecular weight of oxidized muscle proteins, along with monoclonal antibody binding to specific proteins, permits identification. For example, a previous study measuring muscle protein content via Western blot analyses has identified the myosin heavy chains as being the predominant 200-kDa protein in skeletal muscle (15). Furthermore, based on the primary structure (i.e., amino acid content), along with the secondary and tertiary structure of myosin, it has been argued that this protein is highly susceptible to oxidation and the formation of carbonyl groups (7). Therefore, on the basis of these results and our findings using a monoclonal antibody against myosin heavy chains, we postulate that the increase in RCD in 200-kDa proteins within diaphragms from MV animals was due to the oxidation of the myosin heavy chain subunit of native myosin.

MV was also associated with significant increases in RCD in diaphragmatic proteins with molecular masses of ~128 kDa. A search of protein structure databases reveals that numerous 128-kDa proteins exist in skeletal muscle, with the most common proteins being membrane-bound proteins or myosin binding proteins. Therefore, due to the large variety of 128-kDa proteins in skeletal muscle, the present study does not permit speculation about the identity of specific 128-kDa proteins that were oxidized in diaphragms from MV animals.

Our results also indicate that MV was associated with significant increases in RCD in diaphragmatic proteins at molecular masses of ~85 kDa. Although several skeletal muscle proteins have molecular masses of ~85 kDa, Radak et al. (21) have argued that the primary ~85-kDa skeletal muscle protein that is oxidized during exercise is aconitase (EC 4.2.1.3). Aconitase is a mitochondrial enzyme that functions in the Krebs cycle (i.e., converts citrate into isocitrate). Furthermore, in vitro experiments reveal that aconitase is particularly sensitive to oxidant stress and the formation of protein carbonyls (34). Nonetheless, based on the present data alone, it is unclear whether aconitase is a primary target of oxidation in the diaphragm during MV.

Finally, MV also resulted in significant increases in RCD in diaphragmatic proteins with molecular masses of ~40 kDa. Several studies have identified the contractile protein actin as the predominant 40-kDa protein in skeletal muscle (6, 15). Importantly, it has also been reported that actin is susceptible to oxidation when exposed to ROS (6). Therefore, due to both abun-

dance and susceptibility to oxidation, actin is a strong candidate for a muscle protein to contain RCD at this molecular weight. Our data using a monoclonal antibody against skeletal muscle actin support this conclusion.

Critique of Experimental Model

Because of 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 (e.g., 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 anatomic features, and function (14, 16).

Many adult patients requiring MV are maintained on some form of pressure-assist MV. To investigate the effects of prolonged MV on diaphragmatic oxidative injury, we chose to use controlled MV, because this mode of MV results in no diaphragmatic contractile activity and because controlled MV has relevance in several clinical situations. For example, controlled MV is used in adult patients in numerous circumstances (e.g., drug overdose, spinal cord injury, and surgery) and is also commonly used in pediatric patients (9).

Finally, we chose pentobarbital sodium as the general anesthetic in these experiments because of direct evidence that this anesthetic does not promote oxidative injury in the diaphragm or negatively impact diaphragm atrophy or contractile function (19, 28, 29).

Conclusions and Future Directions

These experiments support the hypothesis that the onset of MV-induced diaphragmatic oxidative injury is rapid and that insoluble proteins are targets for oxidative modification. Indeed, MV-induced protein oxidation was evident in insoluble proteins in the diaphragm with molecular masses of 200, 128, 85, and 40 kDa. Although our experiments do not reveal the identity of all of the specific proteins that are oxidized during MV, based on antibody identification, we postulate that diaphragmatic actin (40 kDa) and myosin (200 kDa) are strong candidates for oxidation during MV. If one or both of these contractile proteins are oxidatively damaged during MV, it is conceivable that this type of oxidative injury could contribute to both the decrease in contractile proteins and the force-generating capacity in the diaphragm observed after MV. This is an interesting possibility and should be addressed in future experiments.

Finally, our data also reveal that oxidative injury in the diaphragm occurs within the first 6 h after the onset of MV. At present, the sources of oxidant production within the diaphragm during MV are unknown. Knowledge about the specific pathways responsible for MV-induced diaphragmatic oxidant production would provide important information to develop countermeasures to reduce or prevent MV-mediated oxidative stress in this primary inspiratory muscle.

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