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Mechanical ventilation induces diaphragmatic mitochondrial dysfunction and increased oxidant production

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Abstract

Mechanical ventilation (MV) is a life-saving intervention used in patients with acute respiratory failure. Unfortunately, prolonged MV results in diaphragmatic weakness, which is an important contributor to the failure to wean patients from MV. Our laboratory has previously shown that reactive oxygen species (ROS) play a critical role in mediating diaphragmatic weakness after MV. However, the pathways responsible for MV-induced diaphragmatic ROS production remain unknown. These experiments tested the hypothesis that prolonged MV results in an increase in mitochondrial ROS release, mitochondrial oxidative damage, and mitochondrial dysfunction. To test this hypothesis, adult (3–4 months of age) female Sprague–Dawley rats were assigned to either a control or a 12-h MV group. After treatment, diaphragms were removed and mitochondria were isolated for subsequent respiratory and biochemical measurements. Compared to control, prolonged MV resulted in a lower respiratory control ratio in diaphragmatic mitochondria. Furthermore, diaphragmatic mitochondria from MV animals released higher rates of ROS in both State 3 and State 4 respiration. Prolonged MV was also associated with diaphragmatic mitochondrial oxidative damage as indicated by increased lipid peroxidation and protein oxidation. Finally, our data also reveal that the activities of the electron transport chain complexes II, III, and IV are depressed in mitochondria isolated from diaphragms of MV animals. In conclusion, these results are consistent with the concept that diaphragmatic inactivity promotes an increase in mitochondrial ROS emission, mitochondrial oxidative damage, and mitochondrial respiratory dysfunction.

Keywords

Mitochondria; Superoxide; Antioxidants; Free radicals

Mechanical ventilation (MV) is a life-saving measure used to maintain alveolar ventilation in patients incapable of doing so on their own (e.g., respiratory failure, coma, or spinal cord injury). Unfortunately, prolonged MV reduces the activity of the principal muscle of inspiration (i.e., diaphragm) and results in diaphragmatic wasting and contractile dysfunction [1–14]. Indeed, MV results in a rapid onset of diaphragmatic atrophy that is accompanied by oxidative stress [1,15,16]. Although extended periods of disuse also lead to locomotor skeletal muscle atrophy [13,17–26], a unique characteristic of MV-induced diaphragmatic atrophy is the rapidity of the atrophic response [25,26]. Although the molecular steps that regulate MV-induced diaphragm atrophy remain unclear, growing evidence indicates that redox disturbances in diaphragmatic fibers play a key signaling role in this process. In this regard, our laboratory was the first to report that prolonged MV results in both protein oxidation and lipid peroxidation

in the diaphragm [13,15]. Specifically, diaphragm unloading via MV is associated with a rapid onset of diaphragmatic oxidative stress that develops within 3–6 h after the initiation of MV [15]. Importantly, antioxidant administration during MV eliminates MV-induced diaphragmatic atrophy and dysfunction [1,27].

Which reactive oxygen species (ROS) or reactive nitrogen species production pathways are the primary contributors to MV-induced diaphragmatic oxidative injury remains unknown. Theoretically, inactivity-induced oxidative damage in diaphragm muscle can occur owing to the interaction of several major oxidant-producing pathways. For example, xanthine oxidase production of superoxide, nitric oxide synthase production of nitric oxide, NADPH oxidase-mediated production of superoxide, and mitochondrial production of superoxide can all contribute to diaphragmatic oxidative damage during MV [28–33]. Nonetheless, data from our laboratory reveal that nitric oxide production via nitric oxide synthase [34] and xanthine oxidase production of superoxide are not the predominant pathways of MV-induced diaphragmatic ROS production [35].

In our ongoing search for the dominant source of MV-induced ROS production in the diaphragm, this study examined the role that mitochondria play in ROS generation in the diaphragm. In particular, these experiments investigated the effects of prolonged MV on diaphragmatic mitochondrial ROS emission and determined whether prolonged MV results in mitochondrial oxidative damage and respiratory dysfunction. Based upon preliminary experiments, we hypothesized that after prolonged MV, diaphragmatic mitochondria would exhibit increased ROS emission, oxidative damage, and reduced oxidative phosphorylation capacity. Our results support this hypothesis as mitochondria isolated from the diaphragm of MV rats exhibited higher rates of ROS release, higher levels of oxidative damage, and a lower respiratory control ratio. Furthermore, our data reveal that the activities of electron transport chain complexes II, III, and IV are depressed in diaphragmatic mitochondria after MV.

Methods

Experimental design

To test the hypothesis that during prolonged MV, mitochondria are a major site of ROS production in diaphragm fibers, adult (3–4 months of age) female Sprague–Dawley rats were randomly assigned to either an acutely anesthetized control group ($n = 8$) or a 12-h MV group ($n = 7$). Experiments were conducted in accordance with the policies contained in the *Guide for the Care and Use of Laboratory Animals* and were approved by the institutional Animal Care and Use Committee.

Control animals were subjected to an acute plane of surgical anesthesia with an intraperitoneal injection of pentobarbital sodium (60 mg/kg body wt). Once a surgical plane of anesthesia was attained, the diaphragm muscle was removed and the costal diaphragm was used for mitochondria isolation. In addition, hind-limb skeletal muscles (i.e., soleus, plantaris, white gastrocnemius, and red gastrocnemius) were excised, and all these hind-limb muscles were combined and used for mitochondria isolation.

Animals randomly selected for MV were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg body wt). After reaching a surgical plane of anesthesia, the animals were tracheostomized utilizing aseptic techniques and mechanically ventilated with a controlled pressure-limited ventilator (Servoventilator 300; Siemens) for 12 h with the following settings: upper airway pressure limit 20 cm H₂O, pressure control level above PEEP 4–6 cm H₂O, respiratory rate 80 bpm, PEEP 1 cm H₂O. An arterial catheter was inserted into the carotid artery for constant measurement of blood pressure, saline infusion, and collection of arterial blood samples. In the current experiments, blood samples were periodically obtained

and analyzed for pH and the partial pressures of O₂ and CO₂ using an electronic blood gas analyzer (GEM Premier 3000; Instrumentation Laboratory, Lexington, MA, USA). If necessary, adjustments were made to the ventilator pressure to ensure that arterial blood gas and pH measures were within the desired ranges. Arterial PO₂ was maintained at > 70 mm Hg throughout the experiment by adjustments in FIO₂ (22–25% oxygen). Anesthesia was maintained over the entire period of MV by continuous infusion of pentobarbital sodium (10 mg/kg body wt/h) via a venous catheter that was inserted into the jugular vein. Body temperature was maintained by use of a recirculating heating blanket. Additionally, heart rate and electrical activity of the heart were monitored via a lead II ECG using needle electrodes placed subcutaneously.

Body fluid homeostasis was maintained via the intravenous administration of 2.0 mg/kg body wt/h 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, intramuscular injections of glycopyrrolate (0.04 mg/kg/2 h) were used to reduce airway secretions during MV (concentration of glycopyrrolate stock was 0.2 mg/ml). On completion of MV, the costal diaphragm was removed and used for mitochondria isolation. In addition, hind-limb skeletal muscles (i.e., soleus, plantaris, white gastrocnemius, and red gastrocnemius) were excised, and all these hind-limb muscles were combined and used for mitochondria isolation.

Isolation of mitochondria

Mitochondrial isolations were performed at 4°C according to the methods of Makinen and Lee [36] with minor modifications. Excised diaphragms and hind-limb skeletal muscles were trimmed to remove fat and connective tissues, weighed, and placed in 10 volumes of solution I (100 mM KCl, 40 mM Tris–HCl, 10 mM Tris base, 1 mM MgSO₄, 0.1 mM EDTA, 0.2 mM ATP, and 2% (wt/vol) free fatty acid bovine serum albumin (BSA), pH 7.40). Diaphragms were then minced with scissors and the mince was homogenized with an Ultra-Turrax (Cincinnati, OH, USA) blender for 15 s at 40% of full power. Protease (trypsin; Sigma Chemical, St. Louis, MO, USA) was added (5 mg/g wet muscle), and the digested mince was mixed continually for 7 min. Digestion was terminated through the addition of an equal volume of solution I. The homogenate was centrifuged at 500 g for 10 min at 4°C to pellet down contractile protein and cellular debris. The supernatant was rapidly decanted through a double layer of cheesecloth and centrifuged at 3500 g for 10 min to pellet down the mitochondrial fraction. The supernatant was discarded and the mitochondrial pellet was resuspended in solution I. The suspension was centrifuged at 3500g for 10 min. The supernatant was again discarded, and the pellet was resuspended in 10 volumes of solution II (similar to solution I, but without BSA). This resuspended pellet was subsequently centrifuged at 3500g for 10 min. The final mitochondrial pellet was suspended in 250 µl of a solution containing (in mM) 220 mannitol, 70 sucrose, 10 Tris–HCl, and 1 EGTA, pH 7.40.

Mitochondrial integrity

We evaluated the integrity of mitochondria isolated from control animals by visually examining their structural integrity via electron microscopy. Briefly, the preparation of mitochondria for electron microscopy involved the following steps. Isolated mitochondria were immersed in 4% paraformaldehyde and 1% glutaraldehyde in Tyrode solution. Fixed samples were pelleted and washed with Tyrode buffer, followed by washing with 0.1 M sodium cacodylate buffer. Then the samples were postfixed with 2% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h at 4°C. The samples then were dehydrated with a graded series of ethanol, followed by a graded ethanol/TAAB resin (TAAB Laboratories Equipment Ltd, Berks, England), and cured in 100% TAAB at 60°C for at least 24 h. Ultrathin sections were collected and put on 200-

mesh copper grids and poststained with 8% uranyl acetate in 50% ethanol and lead citrate. Sections were examined and images were acquired with a Hitachi H-7600 transmission electron microscope.

Mitochondrial respiration

Mitochondrial oxygen consumption (J_o) was measured as described by Messer et al. [37]. Briefly, J_o was measured polarographically in a respiration chamber maintained at 37°C (Hansatech Instruments, UK). Isolated mitochondria were incubated with 1 ml of respiration buffer adapted from Wanders et al. [38] (100 mM KCl, 50 mM Mops, 20 mM glucose, 10 mM K_2PO_4 , 10 mM $MgCl_2$, 1 mM EGTA, and 0.2% BSA, pH 7.00) at 37°C in a water-jacketed respiratory chamber with continuous stirring (Hansatech Instruments). For State 3 respiration, 2 mM pyruvate and 2 mM malate were used as complex I substrates in the presence of 0.25 mM ADP, and State 4 respiration was recorded following the phosphorylation of ADP as described by Estabrook [39]. In addition, for State 3 respiration, 5 mM succinate was used as a complex II substrate in the presence of 0.25 mM ADP, and State 4 respiration was recorded following the phosphorylation of ADP as described by Estabrook [39].

Mitochondrial ROS production

Mitochondrial ROS production was determined using Amplex red (Molecular Probes, Eugene, OR, USA). Specifically, this assay was developed on the concept that horseradish peroxidase catalyses the H_2O_2 -dependent oxidation of nonfluorescent Amplex red to fluorescent resorufin red. Superoxide dismutase (SOD) was added at 40 units/ml to convert all superoxide into H_2O_2 . We monitored resorufin formation (Amplex red oxidation by H_2O_2) at an excitation wavelength of 545 nm and an emission wavelength of 590 nm using a multiwell plate reader fluorometer (SpectraMax, Molecular Devices, Sunnyvale, CA, USA). We recorded readings of resorufin formation every 5 min for 30 min, and a slope (i.e., rate of formation) was produced. The slope obtained was converted into the rate of H_2O_2 production with a standard curve. The assay was performed at 37°C in 96-well plates using succinate. Importantly, mitochondrial ROS production was measured in both “resting” (State 4; no ADP added) mitochondria and those at approximately 50% of maximum State 3 respiration by utilizing a novel creatine kinase energy clamp technique to maintain respiration at a steady state. Methodological details of the creatine kinase energy clamp have been described previously by Messer and collaborators [37].

Measurement of reactive carbonyl derivatives

Oxidized proteins in diaphragm mitochondria were quantified by Western blot by using a commercially available kit (Oxy-Blot protein oxidation detection kit; Intergen, Purchase, NY, USA). The carbonyl groups in the protein side chains of both soluble and insoluble proteins were derivatized to 2,4-dinitrophenylhydrazone (DNP). These DNP-derivatized protein samples were separated by using sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After electrophoresis, the proteins were transferred to nitrocellulose membranes. The resulting membranes were then stained with Ponceau S and analyzed to verify equal loading and transfer. Membranes were blocked (2 h) with 5% skim milk in phosphate-buffered saline solution containing 0.05% Tween 20 (PBST). Blots were then incubated in blocking buffer with antibody specific to the DNP moiety of the proteins overnight at 4°C. After being washed with PBST, the blots were incubated at room temperature for 1 h with the appropriate secondary antibody coupled to horseradish peroxidase and washed again with PBST. The membranes were then treated with chemiluminescence reagents (luminol and enhancer; Amersham Biosciences, Pittsburgh, PA, USA) and exposed to light-sensitive film. Images of these films were captured and analyzed using the 440CF Kodak Imaging System (Kodak, New Haven,

CT, USA). The oxidative status of samples was determined by comparing the signal intensity of the entire lane.

4-Hydroxynonenal-modified proteins

4-Hydroxynonenal (4-HNE; *trans*-4-hydroxy-2-nonenal, C₉H₁₆O₂) is a β -unsaturated hydroxyalkenal that is produced as a result of cellular lipid peroxidation. Therefore, 4-HNE was analyzed as an indicator of mitochondrial oxidative stress via Western blotting. Mitochondrial proteins were separated by performing sodium dodecyl sulfate–polyacrylamide gel electrophoresis and subsequently transferred to nitrocellulose membranes. Membranes were blocked (2 h) with 5% skim milk in PBST. Blots were then incubated in blocking buffer with antibody directed against 4-HNE (ab46545; 1:1000 dilution; Abcam, Cambridge, MA, USA). After being washed with PBST, blots were incubated at room temperature for 1 h with secondary antibody coupled to horseradish peroxidase and washed again with PBST. The membranes were then treated with chemiluminescence reagents (luminol and enhancer; Amersham Biosciences) and exposed to light-sensitive film. Images of these films were captured and analyzed using the 440CF Kodak Imaging System (Kodak).

Immunoblotting for antioxidant enzymes

Isolated mitochondrial protein extracts were separated by performing sodium dodecyl sulfate–polyacrylamide gel electrophoresis and subsequently electroblotted onto nitrocellulose membranes. The resulting membranes were then stained with Ponceau S and analyzed to verify equal loading and transfer. Membranes were blocked (2 h) with 5% skim milk in PBST. Blots were then incubated in blocking buffer with antibody directed against copper/zinc superoxide dismutase (SOD1; sc11407; 1:1000 dilution), manganese superoxide dismutase (SOD2; sc30080; 1:1000 dilution), catalase (ab16731, 1:1000 dilution), and glutathione peroxidase 1 (GPX1; ab22604; 1:1000 dilution) overnight at 4°C. Antibodies directed against SOD1 and SOD2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and antibodies directed against catalase and GPX1 were purchased from Abcam. After being washed with PBST, blots were incubated at room temperature for 1 h with the appropriate secondary antibody coupled to horseradish peroxidase and washed again with PBST. The membranes were then treated with chemiluminescence reagents (luminol and enhancer; Amersham Biosciences) and exposed to light-sensitive film. Images of these films were captured and analyzed using the 440CF Kodak Imaging System (Kodak).

Enzymatic assays for electron transport chain complex activity

Before analysis, diaphragm mitochondria were subjected to three cycles of freezing and thawing to lyse membranes. Complex I (NADH dehydrogenase) enzyme activity (EC 1.6.5.3) was measured as a function of the decrease in absorbance from NADH oxidation by decylubiquinone before and after rotenone addition [40]. Complex II (succinate dehydrogenase) activity (EC 1.3.5.1) was measured as a function of the decrease in absorbance from 2,6-dichloroindophenol reduction [40]. Complex III (ubiquinol cytochrome *c* oxidoreductase) activity (EC 1.10.2.2) was determined as a function of the increase in absorbance from cytochrome *c* reduction [40]. Complex IV (cytochrome *c* oxidoreductase) activity was determined as a function of the decrease in absorbance from cytochrome *c* oxidation [40]. Specificity of complex IV activity was determined by monitoring changes in absorbance in the presence of KCN [40]. Citrate synthase (EC 4.1.3.7) was measured as a function of the increase in absorbance from 5,5'-dithiobis-2-nitrobenzoic acid reduction [40]. Enzyme activities are expressed as a ratio to citrate synthase to compensate for mitochondrial enrichment in the cell samples.

Statistical methods

Statistical significance between groups for dependent variables was determined by a one-way analysis of variance. Significance was established at $p < 0.05$. Results are presented as means $\pm$ standard error of the mean.

Results

Diaphragmatic mitochondrial oxidative phosphorylation

We evaluated the suitability of our isolation of mitochondria by visually examining their structural integrity and lack of impurities via electron microscopy. Importantly, images of isolated mitochondria obtained with transmission electron microscopy reveal that our isolation procedure produced high levels of intact and pure mitochondria (Fig. 1). Furthermore, respiratory control ratio (RCR) data from control animals indicate that our isolation procedure yielded well-coupled mitochondria. Mechanical ventilation did not alter the rate of State 3 respiration in diaphragmatic mitochondria (Fig. 2A). However, mitochondria isolated from MV diaphragms exhibited a significant increase ($p < 0.05$) in the rate of State 4 respiration when pyruvate and malate were used as substrate (Fig. 2B). In addition, when succinate was used as a substrate, the rate of State 4 respiration was significantly increased ($p < 0.05$) in diaphragm mitochondria isolated from MV animals compared to controls (Fig. 2B). As a result, the RCR of diaphragmatic mitochondria was significantly reduced after MV when both pyruvate/malate and succinate were used as substrates (Fig. 2C).

Limb-muscle mitochondrial oxidative phosphorylation

We also isolated mitochondria from hind-limb skeletal muscles (i.e., soleus, plantaris, white gastrocnemius, and red gastrocnemius combined) from MV and control animals. Our results indicate that hind-limb skeletal muscle mitochondria are not altered as a result of 12 h of MV. Specifically, when pyruvate and malate were used as a substrate, no significant changes were observed in State 3, State 4, or RCR in mitochondria isolated from limb muscles of control or MV animals (Figs. 3A–C). When succinate was used as a substrate, both State 3 and State 4 were significantly increased ($p < 0.05$) in mitochondria isolated from limb muscles of MV animals. However, RCR was not significantly different between the two groups (Figs. 3A–C).

Mitochondrial ROS production from diaphragm and hind-limb skeletal muscles

Most studies report mitochondrial ROS production under basal conditions (i.e., no ADP present). However, recently it has been shown that mitochondria produce higher levels of ROS in State 4 compared to State 3 [41,42]. Therefore, we determined the rates of mitochondrial ROS production using a complex II-linked substrate (i.e., succinate) in both State 3 and State 4. In diaphragm mitochondria, MV significantly increased ($p < 0.05$) ROS production compared to controls in both State 3 and State 4 (Fig. 4A). In contrast, our data indicate that ROS production from hind-limb skeletal muscle mitochondria was not significantly different between control and MV animals (Fig. 4B). Importantly, in agreement with previous studies, our data indicate that both diaphragmatic and hind-limb skeletal muscle mitochondria produce higher rates of superoxide under State 4 conditions (i.e., in the absence of ADP) compared to State 3 conditions (ADP present).

Lipid hydroperoxides are elevated in diaphragmatic mitochondria after MV

The development of lipid peroxidation in mitochondria isolated from the diaphragm was determined by measuring β -unsaturated 4-HNE-modified proteins. Representative blots are shown in the top of Fig. 5A, and computerized image analysis revealed that 12 h of MV resulted in significant increases ($p < 0.05$) in 4-HNE (Fig. 5A).

MV results in elevated reactive carbonyl derivatives in diaphragmatic mitochondria

Reactive carbonyl derivatives were measured as a marker of protein oxidation in diaphragmatic mitochondria isolated from both experimental groups. Fig. 5B presents representative Western blots illustrating the reactive carbonyl derivatives content in the isolated diaphragmatic mitochondria in control animals and animals exposed to MV. Computerized image analysis of these blots revealed that 12 h of MV resulted in significant increases ($p < 0.05$) in reactive carbonyl derivatives in diaphragmatic mitochondria (Fig. 5B).

Mitochondrial protein content of antioxidant enzymes

The diaphragmatic mitochondrial antioxidant proteins assessed were GPX1, SOD1, SOD2, and catalase. No significant change in protein levels of SOD1 or SOD2 was detected between the two groups (Figs. 6A and B). However, catalase (Fig. 6C) and GPX1 (Fig. 6D) were significantly higher ($p < 0.05$) in the MV diaphragmatic mitochondria compared to controls.

Abnormal electron transport chain complex activities in mitochondria isolated from MV diaphragms

The activities of diaphragmatic mitochondria complexes I, II, III, and IV were evaluated by spectrophotometric methods. The activity of complexes II, III, and IV in diaphragm mitochondria isolated from MV animals was lower compared to control ($p < 0.05$) (Fig. 7). Specifically, the activity of complex II (Fig. 7B) was reduced by 37% in diaphragm mitochondria isolated from MV animals compared to control ($p < 0.05$). Furthermore, diaphragmatic MV mitochondria exhibited a 31% reduction in the activity of complex III (Fig. 7C) compared to diaphragmatic control mitochondria ($p < 0.05$). Moreover, the electron transport chain complex IV activity (Fig. 7D) of MV diaphragmatic mitochondria was reduced by 25% compared to control ($p < 0.05$).

Discussion

Overview of principle findings

This investigation provides several new and important findings. First, our results reveal that prolonged MV results in increased mitochondrial ROS emission and uncoupling of diaphragmatic mitochondria. Our data also indicate that prolonged MV results in depressed activity of electron transport chain complexes II, III, and IV in diaphragmatic mitochondria. Collectively, these findings are consistent with the concept that mitochondria are an important source of ROS release in the diaphragm during prolonged MV. This is important because previous work has demonstrated that oxidative damage to the diaphragm plays a critical role in MV-induced diaphragmatic atrophy and contractile dysfunction [1–14]. A detailed discussion of these and related issues follows.

Prolonged MV results in increased diaphragmatic mitochondrial ROS production

Mechanical ventilation is used clinically to maintain adequate alveolar ventilation in patients incapable of doing so on their own. Although MV is a life-saving measure for patients suffering from respiratory failure, MV results in the rapid onset of diaphragmatic oxidative stress that promotes both diaphragmatic atrophy and contractile dysfunction [1–14]. Indeed, prevention of MV-induced oxidative stress in the diaphragm has been shown to protect the diaphragm against both atrophy and contractile dysfunction [1,27]. Therefore, determining the sources of ROS production in the diaphragm during MV is important. The current study tested the hypothesis that mitochondria are a major source of ROS emission in the diaphragm during prolonged MV. Our results clearly support this postulate, as mitochondria isolated from diaphragms of MV animals released significantly more ROS in both active State 3 and basal State 4 respiration compared to mitochondria isolated from diaphragms of control animals.

Moreover, our findings indicate that prolonged MV results in a significant increase in biomarkers of oxidative damage (e.g., 4-hydroxynonenal and protein carbonyl levels) in isolated diaphragmatic mitochondria.

Historically, it has been postulated that basal mitochondrial respiration (State 4) elicits extremely low levels of H_2O_2 production in skeletal muscle fibers [43]. However, the current study and two recent reports [41,42] using a new and highly sensitive fluorescent indicator of H_2O_2 (Amplex red) show that skeletal muscle mitochondria generate significantly more ROS in State 4 respiration compared to State 3 respiration. Indeed, in the current experiments, diaphragmatic mitochondria released more than 10-fold higher levels of H_2O_2 in State 4 respiration compared to State 3. This finding has great physiological relevance to the current experiments. Indeed, during normal breathing the diaphragm is chronically active (breathing frequency in rat 80 breaths/min) and therefore diaphragmatic mitochondria will be highly active and functioning in State 3 respiration. In contrast, during prolonged MV the diaphragm would become inactive and diaphragmatic mitochondria would shift from State 3 respiration to basal State 4 respiration, resulting in significantly higher levels of mitochondrial ROS release.

Our technique for assessing mitochondrial superoxide production does not measure superoxide directly but evaluates H_2O_2 release from mitochondria and, therefore, provides a means of estimating total superoxide production. It follows that differences in diaphragmatic mitochondrial H_2O_2 scavenging capacity between our control and MV groups would influence the magnitude of H_2O_2 release in our system. Therefore, to compare the diaphragmatic mitochondrial ROS scavenging capacity between the experimental groups, we measured the protein abundance of four primary mitochondrial antioxidant enzymes. In this regard, SOD can dismutate superoxide anions to hydrogen peroxide, and catalase can provide protection against oxidative injury by converting hydrogen peroxide to water and oxygen. Similarly, GPX1 utilizes reduced glutathione as a reducing equivalent to reduce hydrogen peroxide to form oxidized glutathione and water. Our findings reveal that prolonged MV did not alter mitochondrial levels of SOD1 and SOD2 in the diaphragm. However, prolonged MV resulted in a significant increase in diaphragmatic mitochondrial levels of both GPX1 and catalase. Therefore, assuming that an increase in protein abundance of these enzymes translates into increased H_2O_2 scavenging capacity, it is predicted that diaphragmatic mitochondria from MV animals would eliminate more H_2O_2 than the control animals. Therefore, our finding that, compared to control, mitochondrial H_2O_2 emission was greater in diaphragms from MV animals indicates that this observation was due to increased mitochondrial ROS production and not a depressed H_2O_2 scavenging capacity in mitochondria from diaphragms of MV animals.

Another point of interest is that the fiber-type composition of the rat diaphragm could influence the magnitude of mitochondrial ROS production during prolonged MV. Indeed, a recent report reveals that type II skeletal muscle fibers possess unique properties that potentiate mitochondrial ROS production [41]. Specifically, Anderson and Neuffer have demonstrated that compared to type I fibers, mitochondria from type II muscle fibers produce significantly higher levels of superoxide [41]. This is relevant to the current study because the rat diaphragm contains approximately 75% type II fibers [44]. Nonetheless, whether the high percentage of type II fibers in the diaphragm is a primary factor that contributes to the MV-induced development of mitochondrial damage and diaphragmatic atrophy remains unknown.

Prolonged MV promotes diaphragmatic mitochondrial damage and uncoupling

Mitochondria are vital organelles and play multifaceted roles in cells. Despite their primary role in energy production, mitochondria also generate ROS that directly or indirectly influence numerous cellular functions. It is well established that in tightly coupled mitochondria, the rate

of electron flow through the respiratory chain is limited by the rate of ATP synthesis and that the rate of electron flow through the respiratory chain complex is constrained by the rate of proton pumping at the specific coupling sites (reviewed in [45]). Our current experiments reveal that mitochondria isolated from the diaphragms of MV animals exhibit decreased coupling (i.e., lower RCR). This impact of MV on skeletal muscle mitochondrial coupling was unique to diaphragm muscle because MV did not alter mitochondrial coupling from locomotor muscles from the MV animals. The explanation for this differential effect between diaphragm muscle and locomotor muscle is unclear and requires further study.

Damage to the mitochondria, especially at complexes I and III, exponentially enhances the production of ROS [46,47]. At complex I, the production of ROS occurs primarily on the matrix side of the inner mitochondrial membrane [48]. In addition to complex I, complex III is regarded as an important site of superoxide production, and ROS produced at this site (i.e., complex III) appear on both sides of the inner mitochondrial membrane [49]. Our data show defects in MV diaphragmatic mitochondrial complex III, which is consistent with the higher ROS emission rates observed from the diaphragmatic mitochondria isolated from the MV animals. Despite the lack of changes detected in MV diaphragm mitochondria complex I activity, it is still possible that higher emission rates of ROS occur at complex I. Determining the major sites of ROS emission using specific inhibitors of each complex is warranted in the future.

Conclusions

During disuse atrophy, skeletal muscle is subjected to oxidative stress. As a result, this redox disturbance is linked to several signaling processes that lead to muscle wasting. Our results support earlier observations that MV leads to diaphragmatic oxidative stress. Importantly, our novel findings clearly demonstrate that mitochondrial ROS production is a major oxidant-generating pathway involved in the development of oxidative stress in MV diaphragms. Given the important clinical ramifications of muscle wasting in both postural and respiratory skeletal muscle, it is critical to develop therapeutic countermeasures to circumvent morbidity and mortality outcomes in patient populations experiencing muscle wasting (e.g., prolonged bed rest, cancer, and MV). In this regard, the present investigation provides insight into the mechanisms responsible for a critical oxidant production pathway (i.e., mitochondria). Importantly, our study provides the basis for future translation studies to develop therapeutic countermeasures (i.e., mitochondria-targeted antioxidants) to retard inactivity-induced skeletal muscle atrophy.

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Fig. 1. Representative transmission electron microscopy image of mitochondria isolated from control diaphragms illustrating structural integrity.

Fig. 2. (A) State 3 respiration, (B) State 4 respiration, and (C) respiratory control ratio (RCR) in mitochondria isolated from diaphragms of control ($n = 8$) and mechanically ventilated (MV12) ($n = 7$) animals. The left illustrates data obtained using pyruvate/malate (PYR/MAL) as substrate, whereas the right shows data obtained using succinate as substrate. Data are presented as means $\pm$ standard error of the mean. * $p < 0.05$.

Fig. 3.

(A) State 3 respiration, (B) State 4 respiration, and (C) RCR in mitochondria isolated from hind-limb muscles of control ($n = 8$) and mechanically ventilated (MV12) ($n = 7$) animals. The left illustrates data obtained using pyruvate/malate (PYR/MAL) as substrate, whereas the right shows data obtained using succinate as substrate. Data are presented as means $\pm$ standard error of the mean. * $p < 0.05$.

Fig. 4. Mitochondrial ROS production from (A) diaphragms of control ($n = 7$) and mechanically ventilated (MV12) ($n = 7$) animals and from (B) hind-limb muscles of control ($n = 7$) and mechanically ventilated (MV12) ($n = 7$) animals. Note that mitochondria isolated from diaphragms of MV12 animals released higher levels of ROS compared to controls in both State 3 and State 4. Data are presented as means $\pm$ standard error of the mean. * $p < 0.05$.

Fig. 5.

(A) The levels of 4-hydroxynonenal in diaphragm mitochondria were analyzed as an indicator of lipid peroxidation via Western blotting. (B) Oxidized proteins in diaphragm mitochondria were quantified by Western blot using the oxy-blot technique. Representative Western blots for 4-hydroxynonenal and protein oxidation levels are shown above the graphs. Note that 12 h of mechanical ventilation (MV12) ($n = 7$) resulted in increased lipid peroxidation and protein oxidation in diaphragmatic mitochondria compared to control ($n = 8$). Data are presented as means $\pm$ standard error of the mean. * $p < 0.05$.

Fig. 6. (A) SOD1, (B) SOD2, (C) catalase, and (D) GPX1 protein levels in mitochondria isolated from diaphragms of control ($n = 8$) and mechanically ventilated (MV12) ($n = 7$) animals. Representative Western blots are shown above the graphs. Data are presented as means $\pm$ standard error of the mean. * $p < 0.05$.

Fig. 7. (A) Complex I, (B) complex II, (C) complex III, and (D) complex IV activity in mitochondria isolated from diaphragms of control ($n = 8$) and mechanically ventilated (MV12) ($n = 7$) animals was evaluated by spectrophotometric methods. Note that complex II, complex III, and complex IV activity was reduced in mitochondria isolated from mechanically ventilated animals compared to controls. Data are presented as means $\pm$ standard error of the mean. * $p < 0.05$.