TROLOX SUPPLEMENTATION DURING MECHANICAL VENTILATION ATTENUATES CONTRACTILE DYSFUNCTION AND PROTEIN DEGRADATION

BY

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This thesis is dedicated to my husband, Chad Betters, and my parents, Jim and Sue Jones, for their love and support.

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Prolonged, controlled mechanical ventilation (MV) results in diaphragmatic atrophy and reduced diaphragmatic force generating ability. To investigate whether an antioxidant, Trolox, could attenuate atrophy and force loss, we tested the hypothesis that Trolox supplementation during MV would reduce protein degradation and contractile impairments of the diaphragm by preventing oxidative damage. Further, we postulated that proteolysis during MV is mediated by the ATP-ubiquitin-dependent proteasomal pathway. Sprague-Dawley rats were anesthetized, tracheostomized, and mechanically ventilated with 21% O_2 for 12 hours. Trolox was intravenously infused in a subset of ventilated animals. These were compared to groups of spontaneously breathing (SB) animals anesthetized for 12 hours, as well as an acutely anesthetized control group. Twelve hours of MV resulted in a 17% decrease in maximal tetanic force compared to

controls. However, Trolox supplementation during MV completely attenuated the loss of maximal force. Proteolysis, measured as the release of free tyrosine from *in vitro* muscle strips, was increased 105% in MV animals compared to CON, but not different between CON and MV animals receiving Trolox. Lastly, the chymotrypsin-like activity of the 20S proteaseome was elevated in the MV animals (+76%), but Trolox attenuated this rise in activity. These data indicate that Trolox supplementation during MV completely attenuates MV-induced contractile dysfunction and proteolysis in the diaphragm.

CHAPTER 1
INTRODUCTION

Weaning patients from a mechanical ventilator is a serious clinical issue. Mechanical ventilation (MV) is characteristically used to maintain alveolar ventilation in patients who are incapable of ventilation on their own. As such, MV is an important life-preserving measure. However, removing patients from the ventilator, also known as weaning, can be difficult in many cases. Weaning procedures account for more than 40% of total MV time in patients who have difficulty weaning from the ventilator (Esteban 1994), suggesting that this is a serious clinical issue. Diaphragmatic weakness, the result of atrophy, is a major cause of difficult weaning. Therefore, the mechanisms underlying the rapid loss of diaphragm mass and strength during periods of MV should be explored.

Background

In many clinical situations, patients are unable to maintain adequate alveolar ventilation. In these cases, MV is necessary for life support. This may occur during acute respiratory failure, surgeries involving general anesthesia, diseases such as sepsis, and with pre-term infants whose lungs and respiratory muscles are not completely developed. Unfortunately, removing a person from MV is not always simple since even short periods of MV can weaken the diaphragm to the point where resumption of normal loading leads to diaphragm fatigue and respiratory failure. The process of gradually weaning patients from a ventilator can result in extended hospital stays, as well as additional costs to the patients, insurance companies, and hospitals.

The mechanism(s) behind MV-induced diaphragmatic weakness are unclear at this time. However, recent research by Powers and colleagues (2002) has reported that MV-induced diaphragmatic dysfunction is intrinsic to the muscle and increases in magnitude with increasing time on the ventilator. Oxidative stress is one potential mediator of MV-induced diaphragmatic dysfunction. Oxidative stress is the result of an imbalance between reactive oxygen species (ROS) production and antioxidant protection (Lawler and Powers 1998), and has been implicated as a contributor in numerous pathological conditions, including atherosclerosis, obstructive lung disease, aging, and fatigue of skeletal muscle. Although ROS are continuously produced in human beings, a balance is generally maintained between ROS production and cellular antioxidant systems. However, periods of stress, whether from trauma, ischemia, infection, etc., lead to an increase in the formation of ROS, which may overwhelm antioxidant systems causing oxidative stress. This oxidative stress can cause lipid peroxidation, damage to DNA and proteins, and cell death.

It is known that critical illnesses like sepsis or adult respiratory distress syndrome can drastically increase the ROS production and lead to oxidative stress in skeletal muscle. This is significant because oxidized proteins are more prone to proteolytic attack and degradation (Grune et al. 1995, Grune et al. 1996, Dean et al. 1997, Nagasawa et al. 1997). A similar mechanism may contribute to protein loss in the diaphragm during MV. During periods of muscle disuse, ROS production has been shown to increase (Kondo et al. 1993a, Kondo et al. 1993b). Further, new data indicate significant increases in lipid peroxidation and protein oxidation in the diaphragms of mechanically ventilated rats (Shanely et al. 2002, Zergeroglu et al. 2003), and a corresponding increase in total *in*

vitro protein degradation (Shanely et al. 2002). Therefore, it seems logical that diaphragmatic weakening during MV-induced unloading may be caused by oxidative damage to contractile proteins leading to heightened proteolytic degradation.

Problem Statement

Since prolonged controlled MV results in a significant loss of diaphragmatic maximal force production (LeBourdelles et al. 1994, Powers et al. 2002, Radell et al. 2002, Sassoon et al. 2002, Yang et al. 2002, Capdevila et al. 2003, Shanely et al. 2003), and is associated with evidence of increased oxidative stress (Powers et al. 2002, Shanely et al. 2002, Zergeroglu et al. 2003), we postulate a causal relationship and will seek to examine the efficacy of antioxidant infusion during MV for the preservation of diaphragm function. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble vitamin E analog, is an effective scavenger for a variety of radicals (Walker et al. 1998). This antioxidant prolongs survival of many cell types exposed to oxyradicals (Wu et al. 1991). Therefore, we will specifically determine whether Trolox supplementation during 12 hours of controlled mechanical ventilation will attenuate MV-induced diaphragmatic contractile dysfunction, oxidative stress, and protein degradation.

Variables in Study

Independent variables. We will manipulate mechanical ventilation and Trolox supplementation.

Dependent variables. We will measure diaphragm contractility, tyrosine release as a measure of degradation, 20S proteasome activity, and markers of oxidative stress such as protein carbonyls.

Control variable. We will only study female Sprague-Dawley rats, so gender is purposely excluded from this study. The animals will be young adult rats (~4 months old), thus maturation and aging effects are excluded from the study.

Extraneous variable. We will not control PO_2 levels in these animals, so hypoxic conditions will not be controlled. Pilot experiments have been conducted to confirm that the MV protocol maintains normal arterial PO_2 and PCO_2 levels. However, the spontaneous breathing animals are expected to be mildly hypoxic and hypercapnic due to the effects of the anesthesia. To assess the potential effects of these conditions on diaphragm function, a separate group of rats will be studied without exposure to MV or spontaneous breathing protocols (pure controls).

Hypotheses

We hypothesize that:

1.) Twelve hours of controlled MV will induce contractile dysfunction in the rat diaphragm compared to controls.

2.) Twelve hours of controlled MV will increase oxidative stress levels in the diaphragm muscle compared to control diaphragms.

3.) Twelve hours of controlled MV will increase the rate of total protein degradation within the diaphragm compared to control diaphragms.

4.) Infusion of Trolox during 12 hours of MV will attenuate the diaphragmatic dysfunction, reduce the rate of protein degradation, and decrease oxidative stress levels compared to controls.

Definition of Terms

Controlled mechanical ventilation (MV). Tracheostomized animals will receive all breaths from the volume-controlled small-animal ventilator (Harvard Apparatus). The tidal volume will be ~1 ml/100 g body weight with a respiratory rate of 80 breaths/min.

Positive end-expiratory pressure of 1 cm H_2O will be used throughout the protocol. Therefore, the diaphragm muscle will be effectively unloaded.

Spontaneous breathers (SB). Animals receiving sham surgeries and 12 h of anesthesia, without controlled MV.

Antioxidant. A compound capable of preventing or delaying damage from oxidative stress.

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). a water-soluble vitamin E analog with antioxidant characteristics.

Reactive oxygen species (ROS). Molecules derived from molecular oxygen that have an unpaired electron in their outer orbital, making them highly reactive.

Oxidative stress. An imbalance between a greater production of reactive oxygen species and reduced antioxidant protection.

Proteolysis. The process whereby proteins are broken down into peptide fragments and amino acids. This occurs through three main pathways: (1) lysosomal proteases (cathepsins), (2) Ca^{2+}-dependent cysteine proteases (calpains), and (3) the ATP-dependent, ubiquitin-proteasome pathway.

Ubiquitin. A protein found in all cell types that acts as a molecular tag when attached to proteins, marking them for degradation by the proteasome.

Tyrosine. An amino acid that is neither synthesized nor degraded in skeletal muscle. The net accumulation of this amino acid, assayed fluorometrically within the incubation buffer, reflects net *in vitro* protein degradation within muscle.

Limitations/Delimitations/Assumptions

Limitations. The invasive nature of this research negates the use of human subjects. A rat model has been chosen to study the diaphragm muscle because of the similarities in structure and function between the rat diaphragm and human diaphragm.

Trolox does not readily dissolve in saline. Addition of sodium hydroxide (NaOH) was necessary to solubilze Trolox. This increased the pH of the Trolox solution well above the physiological range. As a result, the spontaneous breathing group receiving Trolox was limited to only 3 animals that survived the 12-h protocol. These animals were not included in statistical analyses, thus we were limited in our ability to control for Trolox infusion without MV.

Delimitations. Gender and species differences may exist in regard to the efficacy of Trolox as a protectant against diaphragmatic dysfunction. We have chosen to study only female Sprague-Dawley rats.

Assumptions. It is assumed that the diaphragm is completely unloaded during MV. Previous experiments have inserted electromyographic (EMG) needles into the muscle and found that it is silent during controlled MV (Le Bourdelles et al. 1994, Powers et al. 2002).

Significance of the Study

Atrophy and protein degradation occurring within an unloaded diaphragm muscle result in an 18% reduction in force generation with just 12 hours of MV (Powers et al. 2002). This force loss increases to 46% with 24 hours of MV. Eighteen hours of MV is associated with a significant increase in lipid peroxidation and protein oxidation (Shanely et al. 2002). This research will improve our knowledge of the mechanisms associated with MV-induced diaphragmatic dysfunction. It will provide insight into clinical

strategies using antioxidants to attenuate diaphragmatic atrophy incurred during MV so that patients may be removed more swiftly and successfully from the ventilator.

CHAPTER 2
LITERATURE REVIEW

The diaphragm is an essential muscle for the maintenance of normal ventilation in mammals. This muscle has an activity level greater than most other skeletal muscles, which puts it at increased risk for atrophy and dysfunction during prolonged periods of inactivity such as mechanical ventilation (MV). The purpose of this study is to determine whether Trolox supplementation during MV will attenuate MV-induced diaphragmatic dysfunction related to oxidative stress and protein degradation. This chapter provides a critical review of the scientific literature related to the proposed project. All pertinent articles in the specified areas will be covered. In some cases, however, the literature abounds and only a few representative articles will be reviewed in detail with cursory reference to other corroborating evidence. Whenever possible, interpretations of the reviewed data will be offered based on perceived consensus in the literature.

This review is organized under the following headings: (a) Skeletal muscle adaptations to unloading, (b) Oxidative stress and skeletal muscle, (c) Skeletal muscle unloading and protein degradation, and (d) Antioxidant supplementation and skeletal muscle.

Skeletal Muscle Adaptations to Unloading

Muscle atrophy, the result of disuse, develops rapidly after immobilization (Appell et al. 1997). Several models of disuse exist that either prevent the loading of skeletal muscle with normal body weight, or eliminate the effect of gravity on the upright position. These models include hindlimb unloading in rats, casting of limbs in animals

and humans, spaceflight or simulation of a microgravity environment, bed rest, and denervation.

Atrophy and Fiber Type Shifts with Unloading

Fast and slow twitch locomotor muscles undergo considerable atrophy with unloading (McDonald and Fitts 1995). Simultaneously, unloaded locomotor muscles exhibit a fiber type conversion from type I to type II fibers (Haida et al. 1989). The greatest change occurs in antigravity, slow twitch muscles such as the soleus. After 1, 2, and 3 wk of hindlimb unloading (HU) in Sprague-Dawley rats, mean mass of the soleus was decreased by 28, 44, and 56%, respectively (McDonald and Fitts 1995). Mean fiber diameter decreased with increasing length of HU. Riley et al. (1990) demonstrated that 14 days of unloading caused a reduction in types I and IIa cross-sectional areas (CSA) of 63 and 47%, respectively. They also showed that HU reduced the muscle-to-body weight ratio showing muscle-specific effects of the unloading treatment.

A decrease in the percentage of slow twitch fibers, with an increase in the percentage of fast twitch fibers, leads to an increase in maximal shortening velocity (Canon and Goubel 1995). A 4 wk hindlimb suspension study led to significant fiber atrophy in both the soleus and extensor digitorium longus (EDL) muscles (Deschenes et al. 2001). A significant decrease in the percentage of type I fibers was noted in unloaded solei. This was accompanied by an increase in the percentages of types IIa and IIx/b fibers. The loss of fiber size is of concern because force production is directly related to fiber size. Muscle unloading failed to induce significant fiber type conversion within the fast twitch EDL muscle.

Like hindlimb unloading, casting, or limb fixation, results in muscle atrophy (Booth 1977, Kondo et al. 1993a, Kondo et al. 1993b, Appell et al. 1997). Both the onset

and the degree of atrophy of limb muscles during casting immobilization are dependent on the length of the muscle during limb fixation (Booth 1977). Booth (1977) performed two sets of experiments using a rat model, one with the ankle and foot fixed in slight plantar flexion (PF), and one with the foot fixed in dorsal flexion (DF). In PF, the calf muscles are slightly less than resting length, while they are slightly lengthened in DF. Weight losses of the gastrocnemius, soleus, quadriceps, and white portion of the vastus lateralis were exponential between days 2-10 of PF immobilization. During 28 days of PF immobilization, the gastrocnemius muscle atrophied by 51%, and the plantaris atrophied by 48%. In contrast, there was no change in the weight of the stretched tibialis anterior muscle during PF immobilization. When the ankle was casted in DF, the calf muscles were stretched beyond resting length and a delay in the onset of atrophy was noted. Appell et al. (1997) reported similar findings. Eight days of casting in male Wistar rats with the soleus muscle in a shortened position lead to a 35% atrophy compared to control muscles. These data clearly demonstrate that a greater degree of atrophy, as well as an earlier onset of atrophy, is seen in muscles fixed in positions that are less than resting length.

Seven days of casting the ankle joint of one hindlimb in the fully extended position resulted in a loss of ~45% of soleus muscle weight (Kondo et al. 1993a). In a similar experiment, 4-, 8-, and 12-day immobilization of one hindlimb by casting led to decreases of 49, 60, and 81% of control soleus muscle weight, respectively (Kondo et al. 1993b). The activity of xanthine oxidase (XOD) increased significantly in the atrophied muscles. Type O (superoxide-producing) XOD was ~2.3 times higher in immobilized muscle than in control muscle. Also, the substrates of XOD, xanthine and hypoxanthine,

increased in atrophy. Therefore, superoxide-generating XOD may be more active in the atrophied muscle, meaning oxidative stress is accompanying atrophy. These observations formed the basis of our original hypothesis that oxidative stress may be associated with diaphragmatic atrophy.

Adaptations in the size, metabolic properties, and vascularity of muscle fibers occur in space where the gravity-dependent load of the body on muscles is absent (Edgerton et al. 1995). Vastus lateralis muscle fibers sampled from astronauts before and after spaceflights showed postflight biopsies with 6-8% fewer type I fibers than preflight. This loss of type I fibers seemed to be accounted for by an increase in type IIa fibers. Mean fiber CSAs were 16-36% smaller after the flight. Little difference in percent atrophy was found in type I versus type II fibers. The number of capillaries per fiber was 24% lower after flight as compared to before flight. Spaceflight resulted in an increase in the myofibrillar ATPase activity of type II fibers, whereas alpha-glycerophosphate dehydrogenase (GPD) activity was 80% higher in type I fibers after flight. This study found significant variability between subjects that was attributed to the volume and kind of physical work that each astronaut performed during flight. The results indicate that the degree of atrophy may have been related to the type of physical activities undertaken during spaceflight.

Contractile Dysfunction with Unloading

Maximal isometric specific tension (Po) of the soleus was significantly reduced after 1 and 2 wk of hindlimb suspension compared to normal loaded solei in Sprague-Dawley rats (McDonald and Fitts 1995). A reduction in the number of cross bridges per fiber area, and possibly a reduced force per cross bridge, may explain the decrease in Po.

The gravity-dependent load of the human body in the upright position is essential for maintenance of lower limb skeletal muscle function (Berg et al. 1997). In particular, contractile properties of slow, antigravity skeletal muscle are sensitive to the microgravity environment. Six days of spaceflight induced contractile changes in the soleus muscles of male Sprague-Dawley rats (Caiozzo et al. 1994). The force-velocity relationship, force-frequency relationship, and fatigability were studied *in situ* 3 h after landing. Maximal isometric tension (Po) was decreased by 24% and maximal shortening velocity was increased by 14% in flight muscles. The flight muscles's force-frequency curve was shifted to the right of the control muscles's curve. Control muscles generated 64% of the initial Po after the fatigue protocol, while flight muscles only generated 36% of initial Po.

Bed rest results in similar changes in skeletal muscle function. After 6 wk of bed rest in 7 healthy men, maximum voluntary isometric and concentric knee extensor torque decreased across angular velocities by 25-30% (Berg et al. 1997). Type I fiber cross-sectional area (CSA) of the vastus lateralis decreased by 18.2%. No change in CSA or fiber diameter was apparent in either type IIa or IIb fibers. The greater loss in strength compared to muscle CSA suggests specific tension of muscle and/or neural input to muscle is reduced. Another study showed that 20 days of bed rest decreased maximal knee extension force by 10.9% (Kawakami et al. 2001). The reduction of muscle strength in this study was likely due to a decreased ability to activate motor units.

In conclusion, antigravity, slow twitch muscles such as the soleus are more susceptible to changes as a result of unloading than are nonpostural, phasic muscles such as the EDL. These changes include fiber atrophy, reduced force production, and a

conversion to a faster type muscle. A decreased number of active cross bridges per volume of muscle after unloading may explain the loss in specific tension seen in humans and animals.

Mechanical Ventilation and Diaphragm Atrophy

An early epidemiological study found that some infants and neonates who had received long-term ventilatory assistance (>12 days) had subnormal diaphragmatic muscle mass on gross necropsy examination (Knisely et al. 1988). The retrospective study examined sections of the costal diaphragm, along with portions of the infrahyoid strap muscle and the posterior portion of the tongue. These extra-diaphragmatic sites appear to be coordinated with diaphragmatic function in infants. Histologic findings in the diaphragms of neonates and infants supported by MV for at least 12 days were consistent with disuse atrophy, denervation atrophy, or failure of normal growth and maturation. Myofibers from the other two sites appeared normal. The researchers concluded that long-term ventilatory assistance predisposes diaphragmatic myofibers to disuse atrophy or to failure of normal growth. This weakening may play a role in difficult weaning procedures from ventilatory support. Thus, mechanical ventilation unloads the diaphragm muscle like hindlimb suspension, casting, spaceflight, bed rest, and denervation unload other major skeletal muscles.

Several studies have shown that controlled MV leads to diaphragmatic atrophy (Le Bourdelles et al. 1994, Shanely et al. 2002, Yang et al. 2002, Capdevila et al. 2003) and reductions in protein content (Shanely et al. 2002). Shanely et al. (2002) observed a significant decrease in both total and costal diaphragm masses after 18 h of MV in rats, but no losses of body mass or soleus mass. All four diaphragmatic myosin heavy chain (MHC) types experienced a reduction in CSA. However, type IIx and IIb fibers atrophied

to a greater extent than type I fibers. This contrasts with results seen in locomotor muscle during periods of atrophy, where type I fibers typically atrophy more than type II fibers.

In rats mechanically ventilated for up to 4 days, a significant decrease in diaphragm weight / body weight was seen, which amounted to a mean reduction of 13.4% compared to controls (Yang et al. 2002). These researchers noted a shift in myosin heavy chain (MHC) isoform from slow-to-fast. There was a decrease in the percentage of fibers expressing type I MHC, while the number of fibers co-expressing both type I and type II MHC increased in the diaphragm (12.5% vs. 3% in controls). In contrast, the percentages of type I, type II, and hybrid fibers remained unchanged in the limb muscles after MV. The combination of mechanical unloading, reduced electrical activity, and intermittent passive shortening in the diaphragm during MV may be powerful stimuli for MHC transformations. These modifications may alter the maximal specific force and fatigue resistance of the diaphragm following MV.

In contrast, Sassoon et al. (2002) found that 3 d of MV did not alter fiber type proportions or their relative contribution to total CSA in a rabbit model. Like Sassoon et al. (2002), Capdevila and colleagues (2003) found no significant alterations in fiber type proportions following 51±3 h in the rabbit diaphragm. It is possible that species differences contributed to the discrepancy between these studies and Yang et al. (2002) since fiber type composition differs in rat and rabbit diaphragms.

Mechanical Ventilation-induced Contractile Dysfunction

Eight studies have been published which confirm that controlled mechanical ventilation alters diaphragm contractile properties. Although different animal models were employed, all of these studies agree that MV significantly reduces diaphragmatic force-generating capacity. One of the earliest studies, performed by Le Bourdelles et al.

(1994), found that 48 h of MV in rats significantly decreased *in vitro* diaphragm contractility compared to controls, while the soleus and EDL muscles's contractility were unaffected. No electrical activity of the diaphragm was detected during MV in the 2 animals in which it was measured. Diaphragmatic force-generating capacity was reduced by 41.5% compared to spontaneously breathing controls. The data did not show a difference in total protein content, or citrate synthase or lactate dehydrogenase enzyme activities between control and MV diaphragms. Their data indicate that the decreased force generation did not result from decreased muscle mass as typically seen with general disuse atrophy. An important finding from this study was that the level of sodium pentobarbital required to maintain a surgical plane of anesthesia over a 2-day period did not induce locomotor muscle atrophy, nor did it impair locomotor muscle maximal tetanic force generation. This is significant because it demonstrates that MV itself exerts deleterious effects on diaphragmatic function independently of anesthesia.

Anzueto et al. (1997) mechanically ventilated adult baboons (n=7) for 11 days and showed that maximum transdiaphragmatic pressure decreased by 25% from day 0 to day 11. In addition, diaphragmatic endurance decreased by 36% from day 0 to day 11. These animals were infused with a long-acting neuromuscular blockade, pancuronium (10 μg/kg/h), which may have contributed to the results. However, pancuronium was withheld on day 11, and reversal of neuromuscular blockade was noted. No major changes occurred in hemodynamics, oxygenation, or lung function. Arterial pressure, pulmonary artery pressure, pulmonary artery occlusion pressure, and cardiac output remained constant over the 11-day period. The absolute force-frequency curves showed a

decrease in diaphragmatic response to all frequencies of stimulation tested. Prolonged MV in this baboon model resulted in impaired diaphragmatic strength and endurance.

Five days of volume-controlled MV in a piglet model resulted in depressed diaphragm contractility and activation (Radell et al. 2002). However, nerve conduction and transmission were unaffected. Bipolar transvenous pacing catheters were used to stimulate the phrenic nerve and pace diaphragm contractions during measurements. The researchers found that transdiaphragmatic pressure (Pdi) decreased over time at all frequencies tested. By day 5, the drop in Pdi was greater than 20% at all frequencies. There was a 30% decrease in compound muscle action potential (CMAP) amplitude of the costal diaphragm from day 1 to day 5. The stable response to repetitive stimulation does not support neuromuscular transmission failure as the cause of dysfunction. Instead, the decrease in CMAP amplitude and fall in force output are indicative of excitation-contraction (E-C) coupling or membrane depolarization as mechanisms leading to diaphragmatic dysfunction. Thus, this study found that nerve conduction and neuromuscular transmission are unaffected during prolonged MV, but the diaphragm does experience a loss in function that may originate at the level of the muscle cell membrane or the contractile apparatus.

Yang et al. (2002) also found that maximal twitch and tetanic force generating capacity were significantly lowered in rats mechanically ventilated for up to 4 days as compared to controls. There were no significant differences in contraction time, half-relaxation time, or fatigue resistance. Optimal muscle length, Lo, was found to be shorter in the MV group compared to controls and anesthetized spontaneous breathers. This may provide indirect evidence for a loss of sarcomeres in series.

A study by Powers et al. (2002) examined the time course of MV-induced diaphragmatic contractile dysfunction in an *in vitro* diaphragm strip preparation. When compared to control rats, MV of 12, 18, and 24 h duration resulted in a right shift in the force-frequency curve of the diaphragm. The magnitude of the curve's right shift was dependent upon the duration of MV. Twelve h of MV resulted in an 18% reduction in the mean diaphragmatic specific tension, while 24 h of MV resulted in a 46% reduction. This experiment also included two groups of spontaneously breathing (SB) animals that were maintained on a surgical plane of anesthesia for 18 and 24 h. Analysis of arterial blood gas tensions and pH revealed that these groups experienced hypoxemia, hypercapnia, and mild acidosis. These disturbances were likely due to hypoventilation resulting from depressed ventilatory drive. Nevertheless, like Le Bourdelles and coworkers (1994), this lab found that the level of pentobarbital sodium required to maintain a surgical plane of anesthesia did not impair *in vitro* diaphragmatic function in the spontaneously breathing animals. The researchers concluded that MV-induced contractile dysfunction was due to intrinsic changes within diaphragm fibers. These may include a reduction in the myofibrillar protein concentration, abnormalities of contractile or cytoskeletal proteins, and/or impaired excitation-contraction (E-C) coupling.

Controlled MV also had a time-dependent deleterious effect on diaphragm contractility in a rabbit model (Sassoon et al. 2002). Transdiaphragmatic pressure decreased to 63% of controls after 1 day of MV, and to 49% after 3 days. Similarly, *in vitro* tetanic force decreased to 86% of control values after 1 day, and to just 44% of controls after 3 days. A major finding of this study was that diaphragm muscle injury accounted for 66% of the variance in the reduction of tetanic force. Significant myofibril

damage was found in diaphragms after 3 days of MV, but not in soleus muscles from the same animals. There was no myofibril damage in control diaphragms. Another study in rabbits mechanically ventilated for 49 ± 1 h also found evidence of altered diaphragm fiber ultrastructure indicative of fiber injury in the MV group (Bernard 2003). Disruption and fragmentation of myofibrils were observed in diaphragms after MV, along with an increase in the size of the interfibrillar space and in the size and number of sarcoplasmic lipid vacuoles. The mechanism for myofibril injury with inactivity is unknown, but may contribute to MV-induced diaphragmatic dysfunction and difficulties in weaning patients from ventilators.

The laboratory of Capdevila et al. (2003) examined both the diaphragm and 5th external intercostal muscle following 51±3 h of MV in rabbits. MV significantly decreased Po compared to controls by 25%. This was significantly worsened after a fatigue run. Diaphragmatic and 5th external intercostal muscle masses were significantly reduced following MV. The MV rabbits had lower peak tetanic tensions, reduced fatigue resistance indices, and increased relaxation times compared to control diaphragms. The force reduction of the diaphragm was most likely related to the change in mass.

Eighteen h of controlled MV in a rat model significantly reduced both diaphragmatic maximal twitch force production and Po (~20%) compared to controls (Shanely et al. 2003). However, diaphragms from MV-treated animals maintained a significantly greater fatigue resistance compared to control animals. That is, MV diaphragms maintained higher relative forces throughout a 30-min fatigue test. When absolute force production was compared, however, the control diaphragms produced higher specific forces than MV diaphragms during the fatigue test. Interestingly, costal

diaphragm citrate synthase, total superoxide dismutase (SOD), Cu-Zn-SOD, and Mn-SOD activities were significantly greater in the MV animals than the control animals. These findings indicate that 18 h of MV improves diaphragmatic fatigue resistance relative to maximal force. However, long duration MV (weeks to months) does impair diaphragmatic endurance.

Oxidative Stress and Skeletal Muscle

Reactive oxygen species (ROS) production results from a number of biochemical reactions, most notably aerobic metabolism (Lawler and Powers 1998). Infection, inflammation, strenuous exercise, and obstructive lung disease are a few conditions that increase diaphragm exposure to ROS.

Skeletal Muscle Atrophy and Dysfunction Related to Oxidative Stress

Kondo et al. (1993a) have found that muscle atrophy induced by immobilization is accompanied by oxidative stress. Thiobarbituric acid reactive substance (TBARS) and oxidized glutathione (GSSG) were increased, while total glutathione (GSH) was reduced in atrophied soleus muscle from immobilized hindlimbs. The ankle of one hindlimb of male Wistar rats was immobilized with the soleus muscle in a shortened position. Some rats were sacrificed after 7 days of immobilization (Atrophy group), while the ankle joints of other rats were remobilized for another 5 days (Recovery group). The TBARS level in atrophic muscle increased significantly in the Recovery group, indicating a rise in lipid peroxidation. GSSG levels were significantly greater in atrophic muscle than that in contralateral control muscle. Total GSH level decreased significantly in atrophic muscle. Both the increase of TBARS and GSSG imply enhanced oxidative stress during muscle recovery from atrophy.

The diaphragm muscle is susceptible to alterations in contractility resulting from a direct effect of hydroxyl radical ($OH^{\bullet}$) and superoxide anion radical ($O_2^{\bullet-}$) on contractile proteins (Callahan et al. 2001). Chemically skinned (Triton X-100) single rat diaphragm fibers exposed to $O_2^{\bullet-}$ had a significant 14.5% reduction in maximum calcium-activated force. Exposure to $OH^{\bullet}$ significantly decreased maximum calcium-activated force by 43.9%. Hydrogen peroxide (H_2O_2) did not affect maximum force or calcium sensitivity. The effects of $OH^{\bullet}$ and $O_2^{\bullet-}$ on contractility may contribute to the characteristic respiratory muscle dysfunction seen in certain pathophysiological conditions such as sepsis and skeletal muscle fatigue.

When proteins are damaged by ROS, their function is impaired, and their susceptibility to proteolysis is enhanced (Nagasawa et al. 1997). Oxidatively modified proteins are easily degraded by the proteasome, a multisubunit proteinase. Rats given an intraperitoneal injection of ferric nitrilotriacetate (FeNTA) and sacrificed at 1.5, 3, and 6 h after injection had significant modification of muscle proteins after an iron overload (Nagasawa et al. 1997). Protein carbonyl content of both soleus and EDL muscles was elevated up to 3 h after injection. These results show that muscle proteins were modified by free radicals generated from the FeNTA injection. The rate of tyrosine release reached a maximum at 3 h after injection, suggesting an increase in the rate of total protein degradation up to 3 h after the onset of an oxidative stress. Myosin and actin responded strongly to specific antibody against 2,4-dinitrophenyl group, implying that these myofibrillar proteins were drastically modified by free radicals. These results suggest that oxidatively modified muscle proteins undergo rapid proteolysis.

MV-induced Oxidative Stress

Shanely et al. (2002) found that oxidative stress, specifically protein carbonyl content and total 8-isoprostane concentration, was increased after 18 h of MV in rats. Protein carbonyl levels significantly increased by 44%, while 8-isoprostane concentration increased 53% compared with controls. Short-term controlled MV of just 6 h was sufficient to increase oxidative injury in the diaphragm of rats (Zergeroglu et al. 2003). Reactive protein carbonyl derivatives (RCD) and lipid hydroperoxides were increased after 6 and 18 h of MV. RCD accumulation was limited to insoluble proteins with molecular masses of ~200, 120, 80, and 40 kDa. Oxidative stress is therefore evident in the diaphragm following MV, and is a potential mediator of MV-induced diaphragmatic dysfunction by way of increased protein degradation.

Skeletal Muscle Unloading and Protein Degradation

In skeletal muscle, the balance between protein synthesis and protein degradation determines whether muscle growth or atrophy will occur (Baracos et al. 1986a, 1986b). Protein degradation, specifically myofibrillar protein degradation, may lead to the dysfunction seen in the diaphragm after MV. Three major pathways exist for general protein degradation: lysosomal proteases, calcium-activated calpains, and the proteasome complex. Many studies indicate that the proteasome is responsible for ~70-80% of the increased cellular protein degradation following an oxidative stress (Grune et al. 1995,1996, Grune and Davies 1997). More specifically, it appears the 20S proteasome is responsible for the degradation of oxidized proteins since the 26S proteasome is inhibited/inactivated by oxidative stress (Reinheckel et al. 1998). Recognition of exposed hydrophobic patches is the proposed mechanism by which the proteasome selectively degrades oxidatively modified proteins (Grune et al. 1997).Oxidative damage to a protein

leads to partial unfolding and exposure of normally shielded internal hydrophobic patches that are recognized by the proteasome, which catalyzes the degradation of that protein.

Net protein degradation can be estimated from the rate of release of free tyrosine from tissue proteins (Lowell et al. 1986). Tyrosine is neither synthesized nor degraded in skeletal muscle, so the net accumulation of this amino acid is directly related to net degradation of cell protein. Tyrosine and 3-methyl histidine (3-MH) release have been used to assess total protein degradation and myofibrillar protein degradation, respectively, under a variety of treatments. For example, Lowell et al. (1986) used both techniques to identify a differential breakdown of myofibrillar and nonmyofibrillar proteins during starvation. Likewise, using a model of denervation atrophy, Furuno et al. (1990) have shown that overall protein breakdown is greater in denervated solei than in contralateral controls. Treatments that block the lysosomal and Ca^{2+}-dependent proteolytic pathways did not attenuate protein breakdown, suggesting proteasome-dependent proteolysis may account for denervation-induced loss of protein. Lastly, muscle length appears important in determining rates of protein degradation (Baracos et al. 1986a). Muscles fixed at resting length (Lo) *in situ* experienced the lowest rate of protein breakdown compared to unrestrained muscles. The unrestrained solei and EDLs shortened spontaneously and had 25-45% greater net protein degradation than muscles fixed at Lo.

Nine days of hindlimb suspension lead to atrophy (-55%), loss of protein (-53%), and elevated protein breakdown (+66%) in rat soleus muscles compared to controls (Taillandier et al. 1996). A non-lysosomal, Ca^{2+}-independent proteolytic pathway accounted for the increased proteolysis and muscle atrophy. This study suggests that

ATP-ubiquitin-dependent proteolysis due to the proteasomal pathway is responsible for the majority of the increased protein degradation and muscle atrophy in unweighted hindlimb muscle.

Eighteen h of MV resulted in significant reductions in diaphragmatic protein content, and significant increases in total *in vitro* protein degradation, as measured by the rate of tyrosine release from diaphragm strips (Shanely et al. 2002). The rate of diaphragmatic protein degradation was increased by 28% after MV compared to controls. The significant increase in diaphragmatic proteolysis following MV could be reduced following the addition of either a proteasome inhibitor (lactacystin) or an inhibitor of both calpain and lysosomal proteases (E64d). These researchers also found that oxidative stress was elevated in the diaphragm following 18 h of MV. Therefore, MV results in an increase in oxidative stress (Shanely et al. 2002) that leads to the oxidation of proteins, making them more susceptible to proteolytic attack and degradation (Grune et al. 1995, Grune et al. 1996, Nagasawa et al. 1997).

Antioxidant Supplementation and Skeletal Muscle

Muscle cells contain complex defense mechanisms to protect against oxidative stress (Powers and Hamilton 1999). The two classes of endogenous protective mechanisms are: 1) enzymatic and 2) nonenzymatic antioxidants. Important enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT). These are responsible for removing superoxide radicals, hydrogen peroxide or organic hydroperoxides, and hydrogen peroxide, respectively. Important nonenzymatic antioxidants include vitamins E and C, beta-carotene, glutathione (GSH), and ubiquinone.

In vitro experiments using excised animal muscle have shown that addition of antioxidants can delay fatigue and improve muscular performance (Shindoh et al. 1990, Reid et al. 1992). For example, N-acetylcysteine (NAC) has been shown to protect an *in situ* rabbit diaphragm strip preparation from oxidative injury during periods of rhythmic repetitive isometric contraction (Shindoh et al. 1990). The researchers postulate that NAC, a potent radical scavenger, may have affected fatigue by preventing free radical-mediated damage in the exercising diaphragm muscle. Similarly, Reid et al. (1992) found improved muscular performance in rat diaphragm fiber bundles with both SOD and CAT supplementation. These antioxidants inhibited low-frequency fatigue, but did not alter high-frequency fatigue.

The effects of antioxidant supplementation on human performance are less definitive. Many studies using human subjects have experimental design weaknesses, and most have only investigated the effects of a single antioxidant rather that combining both lipid-soluble and water-soluble antioxidants (Powers and Hamilton 1999). Few studies show improved human exercise performance with antioxidant supplementation. However, the laboratory of Reid and colleagues (1994) has shown that NAC administration in human subjects improves muscular endurance during low-frequency electric stimulation. During fatiguing contractions at 10 Hz, NAC increased force production by ~15%. However, NAC had no effect on fatigue induced by 40 Hz stimulation, or on recovery from fatigue. Additional research is required to determine the specific effects of supplemental antioxidants on humans. Careful research design and understanding of bioavailability are essential to draw conclusions.

Vitamin E

The most widely studied antioxidant is vitamin E, or alpha-tocopherol (Powers and Hamilton 1999). Kondo et al. (1993a) injected either vitamin E or placebo one time daily into Wistar rats with one hindlimb immobilized for 7 days with the soleus muscle in a shortened position. The TBARS level of atrophic muscle in the vitamin E group was significantly less than in the placebo group. The muscle weight was significantly greater, and the degree of atrophy was significantly reduced by ~20% in the vitamin E group compared to the placebo group. Intraperitoneal injections of vitamin E during periods of muscle atrophy effectively served as an antioxidant to reduce oxidative stress and prevent muscle atrophy.

Eight days of immobilization led to a 35% atrophy in the hindlimb of rats (Appell et al. 1997). However, when vitamin E was supplemented, the muscles atrophied by only 12%. Control muscles of those animals supplemented with vitamin E contained even less of the oxidized form of glutathione (GSSG) than baseline oxidative stress. These results indicate that the soleus muscle atrophies to a lesser extent when supplemental vitamin E is given during a period of disuse.

Trolox

Vitamin E is a natural antioxidant, but is extremely lipophilic and is taken up slowly by cells (Zeng et al. 1991). It is therefore not an adequate therapeutic antioxidant. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), is a hydrophilic vitamin E analog synthesized in 1974 by Scott and colleagues (1974). Trolox differs from vitamin E by the absence of the phytyl side chain, which makes Trolox water-soluble (Klein et al. 1991).

Trolox is an effective scavenger of radicals (Walker et al. 1998). This antioxidant has been shown to prolong the survival of cells exposed to oxyradicals (Wu et al. 1990, Wu et al. 1991). Specifically, it was found that Trolox protected human ventricular myocytes and hepatocytes against oxyradicals generated by xanthine oxidase-hypoxanthine and prevented lysis of erythrocytes exposed to an azo-inhibitor (Wu et al. 1990). The protection by Trolox was dose dependent in all cell types, and surpassed the antioxidant capabilities of ascorbic acid, SOD, and CAT. Using hepatocytes, the researchers determined that Trolox behaved mechanistically as an antioxidant in cells.

In cultured rat hepatocytes, 0.5-16 mmol/L Trolox prolonged the survival of cells exposed to xanthine oxidase-hypoxanthine oxyradicals (Wu et al. 1991). Optimum levels of Trolox were between 1 and 2 mmol/L. Protection by Trolox surpassed that provided by ascorbate, mannitol, SOD, and CAT. This laboratory also studied a global and partial model of hepatic ischemia-reperfusion in rats (Wu et al. 1991). Infusion of Trolox (7.5-10 μmol/kg body weight) prior to reflow reduced liver necrosis by more than 80% compared to control, untreated animals. These data indicate a strong and rapid antioxidant-like action by Trolox on rat hepatocytes and postischemic-reperfused rat liver.

Trolox also protected regionally ischemic, reperfused porcine hearts against free radical generation (Klein et al. 1991). Specifically, Trolox reduced free radical generation from stimulated neutrophils by 30% in the treatment group before ischemia and immediately before reperfusion. After 3 days of reperfusion, recovery of regional function had improved to a significantly greater extent in the treated group than in control hearts. Mean recovery of systolic shortening amounted to 10% of the baseline value in

the control animals, and to 28% in the Trolox group. Trolox did not reduce infarct size, but did accelerate functional recovery in ischemic, reperfused porcine hearts.

Trolox has also been shown to improve the long-term storage of isolated skeletal muscle (van der Heijden et al. 2000). Significant protection of contractile function occurred with addition of 1mM Trolox in the bathing solution of soleus and cutaneus trunci muscles from the rat. These muscles were stored for 16 h at 4^{o}C. Trolox effectively reduced the overproduction of oxyradicals.

Trolox treatment *in vivo* protected methylmercury (MeHg)–treated rat skeletal muscle from many of the clinical manifestations of MeHg-intoxication (Usuki et al. 2001). Trolox prevented decreases in mitochondrial enzyme activities in soleus muscle, repressed apoptosis in cerebellum, and protected against the decrease in glutathione peroxidase activity of the soleus following MeHg-intoxication.

Summary

The removal of weight bearing from skeletal muscle leads to rapid and significant atrophy. The rat model of mechanical ventilation effectively unloads the diaphragm muscle, thereby causing atrophy. Oxidative stress is evident in unloaded muscle, including diaphragm muscle from mechanically ventilated animals, and may increase rates of protein degradation. Interventions with antioxidants have shown that muscle atrophy and dysfunction can be attenuated during unloading. The antioxidant properties of Trolox make it an appealing subject for research. No studies have given Trolox to animals to prevent muscle dysfunction during unloading. This project will determine whether mechanical ventilation-induced diaphragmatic dysfunction, oxidative stress, and proteolysis can be attenuated with supplementation of the antioxidant-like compound, Trolox.

CHAPTER 3
METHODS

Experimental Design

The following groups were formed to complete these experiments:

Pure control group	CON	n=8
Spontaneous breathers	SBS	n=8
Mechanical ventilation	MVS	n=8
Spontaneous breathers receiving Trolox	SBT	n=3
Mechanical ventilation receiving Trolox	MVT	n=8

Animals

The subjects were adult (~4 month-old) female Sprague-Dawley rats (~250 g). All were housed in the J. Hillis Miller Animal Science Center and fed the same diet (rat chow and water *ad libitum*) for one week prior to the experiment. Animals were maintained on a 12 h light:dark photoperiod. All procedures followed NIH guidelines and were approved by the University of Florida's Animal Care and Use Committee.

General Procedures

After a period of acclimation (1 week), rats were randomly assigned to one of the 5 groups listed above. The control group (CON) animals were free of intervention before removal of the diaphragm for measurements. These animals received an acute intraperitoneal injection of sodium pentobarbital (65 mg/kg body weight). When a surgical plane of anesthesia was reached, the diaphragms were removed for measurement of *in vitro* contractile properties and tyrosine release, and remaining muscle was frozen for biochemical assays.

Animals in the 2 mechanically ventilated groups (MVS and MVT) were given an intramuscular injection of glycopyrollate (0.04 mg/kg) to reduce respiratory secretions during the protocol. Thirty min later, animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (65 mg/kg). Upon reaching a surgical plane of anesthesia (no ocular response, no hindlimb withdrawal response), they were tracheostomized by an experienced lab technician and mechanically ventilated with a volume-cycled ventilator (Harvard Apparatus). The tidal volume was established at ~1 ml/100 g body weight with a respiratory rate of 80 breaths/min. Positive end-expiratory pressure of 1 cm H_2O was used for all MV animals. Throughout the MV period, heart activity, blood pressure, and core temperature were monitored. A lead II ECG displayed electrical activity of the heart. A catheter placed in the carotid artery gave constant blood pressure readings. Core temperature was monitored with a rectal thermometer and adjustments were made to help animals maintain body temperature at $37 \pm 1^{o}C$ with a re-circulating heating blanket.

A catheter was placed in the jugular vein for the infusion of sodium pentobarbital (~10 mg/kg/h) and Trolox. In the MVT group, the jugular vein was cannulated before the carotid artery and before the tracheotomy. A priming dose of Trolox (20 mg/kg) was infused over a 5 min period. Twenty min later, MV was started, along with the constant infusion of Trolox at a rate of 5 mg/kg/h. Constant supervision was provided for the rats throughout the MV period. This included expressing the bladder, removing airway mucus, monitoring anesthesia rate, rotating the animals, and infusing saline to maintain hydration status. To reduce airway secretions, glycopyrollate (0.04 mg/kg) was injected intramuscularly every 2 h.

Spontaneously breathing (SBS and SBT) animals were anesthetized in the same manner and received sham surgeries. These animals were included in the study to determine whether long-term anesthesia (sodium pentobarbital) impairs diaphragmatic contractile function. A tube was inserted into the trachea, and these animals were maintained on a surgical plane of anesthesia for the 12 h period while continuing to breathe on their own. The carotid artery and jugular vein were cannulated, and sodium pentobarbital was infused for a 12 h period. However, these animals were not mechanically ventilated. The SBT group received the same dose of Trolox as the MVT group (20 mg/kg priming dose, 5 mg/kg/h constant infusion).

At the end of the 12 h experimental period, all rats were killed by injection of sodium pentobarbital (50 mg/kg) and the diaphragm was removed for immediate analyses of contractile function and protein degradation as described below. After obtaining muscle strips for contractile and protein degradation measurements, the remaining costal diaphragm tissue was dissected, weighed, frozen in liquid nitrogen, and stored at –80^{o}C until needed.

Diaphragm Contractile Function

The entire diaphragm with the supporting ribs and central tendon was removed and placed in a dissecting chamber containing a Krebs-Hensleit solution aerated with 95% O_2-5% CO_2 gas. The entire crural diaphragm was removed and discarded. A strip was cut from the midcostal region including the central tendon and the rib. This was secured vertically in an organ bath maintained at 24^{o}C between two Plexiglass clamps. The muscle strip was placed between two platinum field electrodes connected to an isometric force transducer (model FT-03, Grass Instruments, Quincy, MA). This strip was mounted on a micrometer to allow for muscle length adjustment. A 15 min equilibration period in

the bath preceded all data collection. During this time, the remaining diaphragm muscle was dissected and sectioned into 9 pieces: 2 dorsal, 6 midcostal, and 1 ventral section. All sections were blotted, weighed, frozen in liquid nitrogen, and stored at –80^{o}C. All further analyses were conducted on midcostal diaphragm sections.

We determined optimal muscle length (Lo), the length that generates maximal twitch force, and used this length throughout the protocol. Lo was found by systematically adjusting the length of the muscle while stimulating it with single supramaximal (~150%) twitches and recording the force generated. Lo was measured (in cm) using calipers.

Peak isometric tetanic tension was measured from a series of three contractions with 2 min of recovery between contractions. The force-frequency relationship was studied by stimulating the muscle strips at 15, 30, 60, 100, 160, and 200 Hz (120 V). Each stimulus was applied for 500 ms, and adjacent stimulus trains were separated by 2 min of rest.

Diaphragmatic fatigability was assessed by monitoring the decrease in force development over time. Each muscle strip was stimulated by unfused tetanic contractions (30 Hz, 250 ms) for 30 min. The duty cycle, or time of muscle contraction compared to muscle rest, was 12.5%. Tension was measured at 0, 1, 2, 5, 10, 15, 20, 25, and 30 min. Fatigue resistance was assessed by the percentage of initial force maintained at the end of the 30 min protocol. After all contractile measurements were made, the diaphragm strip was removed from the organ bath. The rib, central tendon, and excess fat and connective tissue were removed from the strip, which was then blotted and weighed. Forces generated were normalized to muscle strip cross-sectional area (CSA).

Protein Degradation

To measure total protein degradation, the release of tyrosine into the incubation medium was measured. Two strips were cut from the midcostal diaphragm (~ 40 mg each). These strips were secured at resting length in separate baths containing Krebs-Ringer bicarbonate solution, which was supplemented with 5 mM glucose, insulin (1 unit/ml), 0.17 mM leucine, 0.10 mM isoleucine, and 0.20 mM valine to improve protein balance, and 5 mM cycloheximide to inhibit protein synthesis. Diaphragm strips were maintained at resting lengths by securing both ends to a solid plexiglass rod. The medium was continuously gassed with 95% O_2 - 5% CO_2. Temperature was maintained at 37^{o}C. Muscle strips were preincubated for 30 min, and then fresh medium was added for a 2 h incubation. After this incubation, the strips were removed, blotted, and weighed. For measurement of tyrosine release, the medium from each bath was aliquoted into microcentrifuge tubes. The aliquots were stored at –20^{o}C until analysis of tyrosine concentration.

Tyrosine in the medium was assayed spectrofluorometrically by the method of Waalkes and Udenfriend (1957) with some modification. Two hundred µl of incubation medium was diluted with 800 µl dH_2O in a glass tube. To this, 0.5 ml of 1-nitroso-2-naphthol reagent (0.1 g 1-nitroso-2-naphthol in 100 ml of 95% methanol) and 0.5 ml of nitric acid reagent (24.5 ml of 20% nitric acid and 0.5 ml of 2.5% $NaNO_2$) were added. The tubes were shaken to mix, and incubated in a water bath at 55^{o}C for 30 min. After cooling for 15 min, 5.0 ml of ethylene dichloride was added to extract the unchanged nitrosonaphthol reagent. The tubes were centrifuged for 15 min at 2500 x g. One ml of the supernatant was transferred to a quartz cuvette and read in a spectrophotofluorometer.

The tyrosine derivative was excited at 460 uM and measured at 570 uM. Standards were prepared using L-tyrosine (Sigma).

20S Proteasome Activity

The chymotrypsin-like activity of the 20S proteasome was measured fluorometrically as the release of AMC from the synthetic substrate Suc-LLVY-AMC. Approximately 50 mg of midcostal diaphragm tissue was homogenized (glass-on-glass) in a homogenizing buffer containing 50 mM Tris base, 1 mM EDTA, 1 mM EGTA, 1 μM Pepstatin-A, 50 μM E-64, and 10% glycerol. This homogenate was centrifuged at 1500 x g for 10 min at 4^{o}C, and the supernatant was then centrifuged at 10,000 x g for 10 min at 4^{o}C. The remaining supernatant was centrifuged at 100,000 x g in an ultracentrifuge for 1 h at 4^{o}C to separate the proteasomal fraction. The resulting supernatant fraction was used to measure protein content using the Bradford method, and to measure proteasome activity. Ten μg of protein was reacted with the synthetic peptide substrate for chymotrypsin-like activity in a reaction mixture containing 50mM Tris-HCl, 1 mM DTT, and 5 mM $MgCl_2$. One aliquot from each sample was incubated with an inhibitor of the chymotrypsin-like proteasomal activity, lactacystin, while the other was not. Samples were incubated for 30 min at 37^{o}C before the addition of substrate. The change in fluorescence was measured at an excitation wavelength of 380 nM and emission of 460 nM. The difference between the activities of the proteasome with and without inhibitor was used as the proteasome activity.

Total and Non-protein Thiols

As an indicator of oxidative stress, we measured total thiol and non-protein thiol groups in diaphragm homogenate. Diaphragm tissue was homogenized in 0.02 M EDTA on ice and centrifuged at 1500 rpm for 10 min at 5 ^{o}C. Twenty-five μl of homogenate was

incubated with 75 µl 0.2 M Tris buffer (pH 8.2), 395 µl methanol, and 5 µl 0.01 M DTNB for 35 min on a bench-top rotator. Samples were centrifuged at room temperature for 15 min at 3,000 x g. Two hundred µl of supernatant was loaded per microplate well (3 wells per sample) and read at a wavelength of 414 nm. A standard curve was generated using glutathione (GSH; Sigma) in 0.02 M EDTA.

Non-protein thiols were measured by incubating 350 µl homogenate with 350 µl 1% metaphosphoric acid for 15 min on a bench-top rotator to precipitate proteins. Samples were centrifuged at room temperature for 15 min at 3,000 x g. Three hundred µl supernatant was then incubated with 200 µl 0.4 M Tris buffer (pH 8.9) and 25 µl 0.01 M DTNB. Tubes were mixed for 10 min and read against GSH standards in a microplate reader at 414 nm.

Limitations

We did not record electromyographic (EMG) activity of the mechanically ventilated diaphragms to ensure that muscle activation was completely suppressed. Powers et al. (2002) performed preliminary experiments where wire electrodes that measure EMG activity were placed in the costal diaphragm of 4 animals during 24 h of controlled MV. No electrical activity was measured in any of these animals during the 24 h procedure. Le Bourdelles and colleagues (1994) did not find EMG activity in the rat diaphragm ventilated for 2 days. Therefore, we assumed that diaphragmatic contractions did not occur during 12 h of controlled MV.

The mortality rate of the SBT group was higher than other groups (~70%). As a result, we were only able to include 3 animals that survived the 12-h spontaneous breathing protocol while receiving Trolox. This made statistical analyses difficult, and conclusions yet uncertain with this group.

Vertebrate Animals

Female Sprague-Dawley rats were used in this research. This study required removal of the diaphragm muscle for analysis, and therefore prevented the use of human subjects. Sprague-Dawley rats were selected since our lab, as well as previous researchers, has successfully used them as subjects in MV studies. The MV experiments were supervised by research assistants who have experience with short-term MV in rats.

Statistical Analysis

This experiment was designed to test the hypotheses that Trolox supplementation during mechanical ventilation would alter diaphragm contractile dysfunction, protein degradation, and oxidative stress. A 4 x 6 (group x stimulation frequency) ANOVA with repeated measures on stimulation frequency was used to analyze the force-frequency data. Likewise, a 4 x 9 (group x time) ANOVA with repeated measures on the time factor was used to analyze data from the fatigue protocol. Where significant differences were found, Tukey's HSD test was implemented post hoc. ANOVAs were used to examine differences between groups for the remaining dependent variables. Independently, the effects of anesthesia were compared using a Student's t-test on SBS and CON group data. Significance was established at $p<0.05$.

CHAPTER 4
RESULTS

Systemic and Biologic Responses to Treatment

The MV protocol did not significantly change body mass for any of the groups (Table 1), indicating that our schedule of nutrition and rehydration was adequate. The ratio of total costal diaphragm mass to final body mass was not significantly different between the 5 groups ($p=0.501$).

There were no signs of infection in any animals, and only 1 MVS animal was eliminated from the study due to evidence of barotrauma to the lungs on post-mortem examination. Systolic blood pressure was maintained at 70-110 mmHg in all groups, and arterial pH, PO_2, and PCO_2 were maintained within physiological ranges for both MV groups. The SB animals were mildly hypoxic, hypercapnic, and acidotic, as expected due to the anesthesia. Body temperature was kept at 37 ± 1^oC during the 12-hour protocol.

Effects of Anesthesia on Diaphragm Contractile Properties

The maximal tetanic force was not different between the SBS and CON groups (25.09 ± 0.41 N/cm^2 vs. 25.33 ± 0.50 N/cm^2, respectively). Likewise, the force-frequency curves and fatigue data were similar between these two groups (Figures 1, 2, and 3), and contractile parameters did not differ (Table 3). Thus, 12 hours of sodium pentobarbital anesthesia did not affect in vitro contractile properties of the diaphragm.

Effects of Mechanical Ventilation on Contractile Properties

Twelve hours of controlled MV reduced maximal tetanic force production by ~17% (21.00 ± 0.71 N/cm^2 vs. 25.43 ± 0.50 N/cm^2 in CON animals). The force-frequency curve

of the MVS group was shifted downward and to the right of the CON group (Figure 1). This indicates a reduction in force generation at all stimulation frequencies tested. The fatigue protocol produced curves of similar shape for all groups (Figure 2), but the MVS group generated a significantly lower amount of force compared to CON, SBS, and MVT groups where indicated. When the fatigue data are expressed as percent of initial force (Figure 3), there are no significant differences between the 4 groups at any time point ($p= 0.230$).

One-half relaxation time (½ RT) of maximal twitch was significantly shorter in the MVS group compared to CON, while ½ time to peak tension (½ TPT), rate of force development, and rate of relaxation were not different for either maximal twitch or maximal tetanic forces (Table 3).

Effects of Trolox on Contractile Properties

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

Twelve hours of Trolox infusion without MV (i.e. the SBT group) greatly increased subject mortality rate. Only three SBT animals survived the treatment. Further, diaphragms from these animals showed impaired force production compared to CON. The force-frequency curve of the SBT group was shifted downward and to the right of the CON group and closely resembles the MVS group (Figure 1). Trolox

supplementation during spontaneous breathing also significantly reduced ½ TPT of maximal tetanic force compared to CON animals.

Protein Degradation

Twelve hours of controlled MV significantly elevated total *in vitro* protein degradation (+105%), as measured by the release of free tyrosine, compared to CON (Figure 4). However, protein degradation of the MVT group (16% increase compared to CON) was not significantly different from CON (p=0.797). There were no significant differences in protein degradation between CON and SBS groups (p=0.351).

20S Proteasome Activity

The chymotrypsin-like activity of the 20S proteasome was significantly increased in the MVS group compared to the CON group (+76%) (Figure 5). Trolox attenuated the MV-induced increase in proteasome activity (+26% compared to CON, p=0.647).

Oxidative Stress

Protein carbonyls and lipid hydroperoxides, two indicators of oxidative stress, were not different between CON, MVS, and MVT groups. However, total thiols were significantly lower in the MVS group as compared to the CON group (134.83 $\pm$ 6.90 nmol/mg protein vs. 157.34 $\pm$ 6.45 nmol/mg,respectively). Similarly, non-protein thiols were significantly lower in the MVS group compared to the CON group (28.02 $\pm$ 1.62 nmol/mg vs. 42.14 $\pm$ 1.51 nmol/mg, respectively). But, Trolox supplementation during MV failed to prevent the loss of total and non-protein thiol groups.

Table 4-1. Body and Diaphragm Weights of Control, Spontaneously Breathing, and Mechanically Ventilated Animals

	CON	SBS	MVS	SBT	MVT
Initial body mass (g)	264.38 ± 5.16	276.88 ± 5.29	282.25 ± 3.33	285.67 ± 2.33	300.13 ± 6.37*†
Final body mass (g)	264.38 ± 5.16	280.56 ± 5.52	286.38 ± 3.18*	292.00 ± 2.00	306.75 ± 6.42*†
Total costal diaphragm mass (mg)	532.57 ± 15.64	567.69 ± 12.86	601.86 ± 14.80	610.63 ± 27.98	632.98 ± 10.74
Total costal diaphragm mass/body mass‡ (mg/g)	2.014 ± 0.04	2.025 ± 0.04	2.100 ± 0.04	2.091 ± 0.09	2.067 ± 0.04

Definition of abbreviations: CON = control animals; SBS = spontaneously breathing animals receiving saline; MVS = mechanically ventilated animals receiving saline; SBT = spontaneously breathing animals receiving Trolox; MVT = mechanically ventilated animals receiving Trolox.
Values represent means ± SEM
* Significantly different from CON group, $p<0.05$.
† Significantly different from SBS group, $p<0.05$.
‡ Mass values expressed as milligrams per gram of body mass were normalized to postexperiment body mass values.

Table 4-2. Maximal Isometric Twitch and Tetanic Force of Control, Spontaneously Breathing, and Mechanically Ventilated Animals

	CON	SBS	MVS	SBT	MVT
Maximal isometric twitch force (N/cm^2)	7.24 ± 0.14	6.79 ± 0.30	5.65 ± 0.28*†‡	5.94 ± 0.55	7.04 ± 0.25
Maximal isometric tetanic force (N/cm^2)	25.43 ± 0.50	25.09 ± 0.41	21.01 ± 0.71*†‡	21.54 ± 1.40*†	25.49 ± 0.50

Definition of abbreviations: CON = control animals; SBS = spontaneously breathing animals receiving saline; MVS = mechanically ventilated animals receiving saline; SBT = spontaneously breathing animals receiving Trolox; MVT = mechanically ventilated animals receiving Trolox.
Values represent means ± SEM
* Significantly different from CON group, $p<0.05$.
† Significantly different from SBS group, $p<0.05$.
‡ Significantly different from MVT group, $p<0.05$.

Table 4-3. Contractile Parameters of Maximal Isometric Twitch and Tetanic Forces of Control, Spontaneously Breathing, and Mechanically Ventilated Animals

	CON	SBS	MVS	SBT	MVT
TWITCH					
½ TPT	0.018 ± 0.000	0.018 ± 0.001	0.017 ± 0.000	0.017 ± 0.000	0.018 ± 0.000
½ RT	0.044 ± 0.001	0.040 ± 0.001	0.034 ± 0.003*	0.037 ± 0.002	0.04 ± 0.001
+ dp/dt	332.29 ± 27.368	420.97 ± 31.176	415.85 ± 57.784	418.82 ± 94.619	459.52 ± 18.567
- dp/dt	-149.26 ± 9.247	-177.84 ± 9.696	-207.93 ± 28.890	-179.61± 34.138	-204.21 ± 7.845*
TETANIC					
½ TPT	0.064 ± 0.002	0.062 ± 0.001	0.056 ± 0.003	0.053 ± 0.003*	0.059 ± 0.002
½ RT	0.064 ± 0.002	0.062 ± 0.003	0.062 ± 0.003	0.061 ± 0.001	0.065 ± 0.002
+ dp/dt	423.71 ± 34.738	527.67 ± 41.175	515.38 ± 95.654	518.80 ± 93.776	570.18 ± 20.668
- dp/dt	-544.55 ± 50.860	-723.04 ± 36.225	-703.53 ± 157.300	-658.41 ± 94.133	-740.20 ± 12.091

Definition of abbreviations: CON = control animals; SBS = spontaneously breathing animals receiving saline; MVS = mechanically ventilated animals receiving saline; SBT = spontaneously breathing animals receiving Trolox; MVT = mechanically ventilated animals receiving Trolox; TPT = time to peak tension; RT = relaxation time; +dp/dt = rate of force development; -dp/dt= rate of force relaxation.
Values represent means ± SEM
* Significantly different from CON group, $p<0.05$.
† Significantly different from SBS group, $p<0.05$.
‡ Significantly different from MVT group, $p<0.05$.

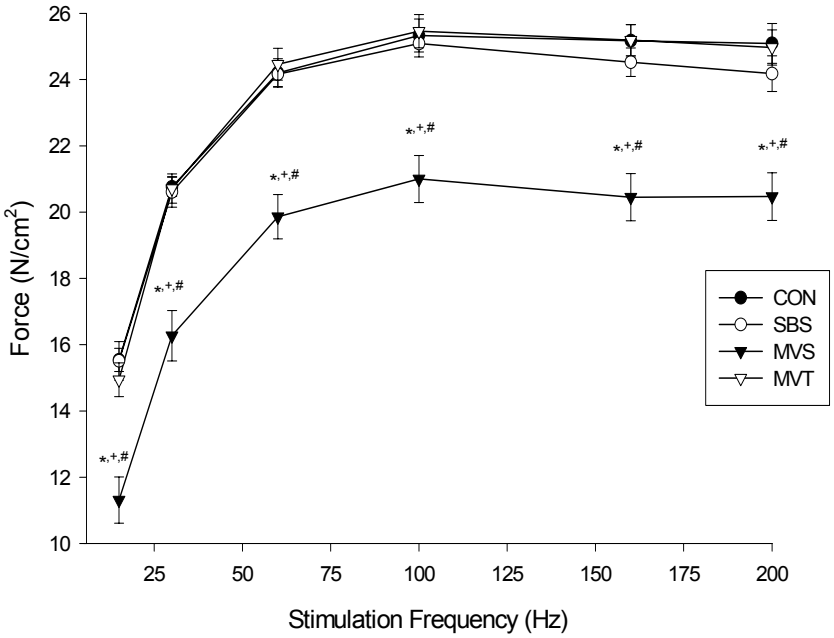

Figure 4-1. Force-frequency responses of control (CON), spontaneously breathing (SBS), mechanical ventilation (MVS), and mechanical ventilation animals receiving Trolox (MVT).

Values represent means ± SEM.
* Significantly different from CON group, $p<0.05$.
+ Significantly different from SBS group, $p<0.05$.
# Significantly different from MVT group, $p<0.05$.

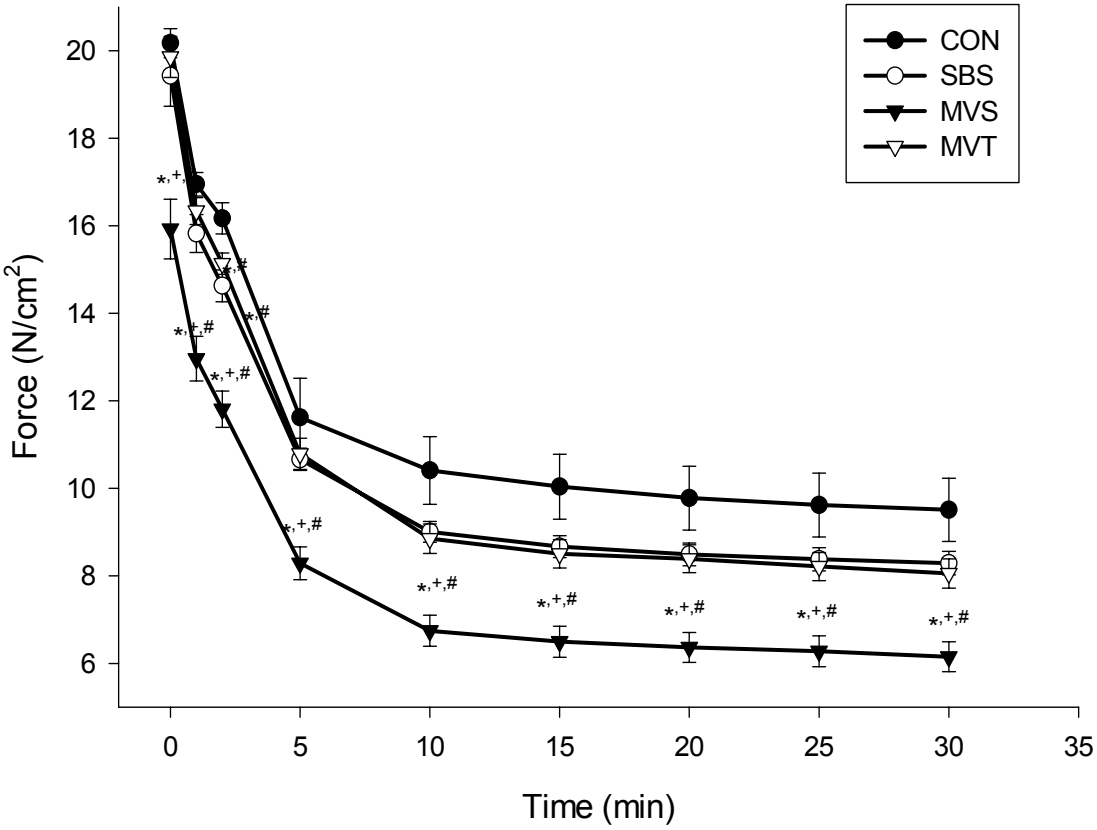

Figure 4-2. Responses of *in vitro* diaphragm strips from control (CON), spontaneously breathing (SBS), mechanical ventilation (MVS), and mechanical ventilation animals receiving Trolox (MVT) to a 30-min fatigue protocol.

Values represent means ± SEM.
* Significantly different from CON group, $p<0.05$.
+ Significantly different from SBS group, $p<0.05$.
# Significantly different from MVT group, $p<0.05$.

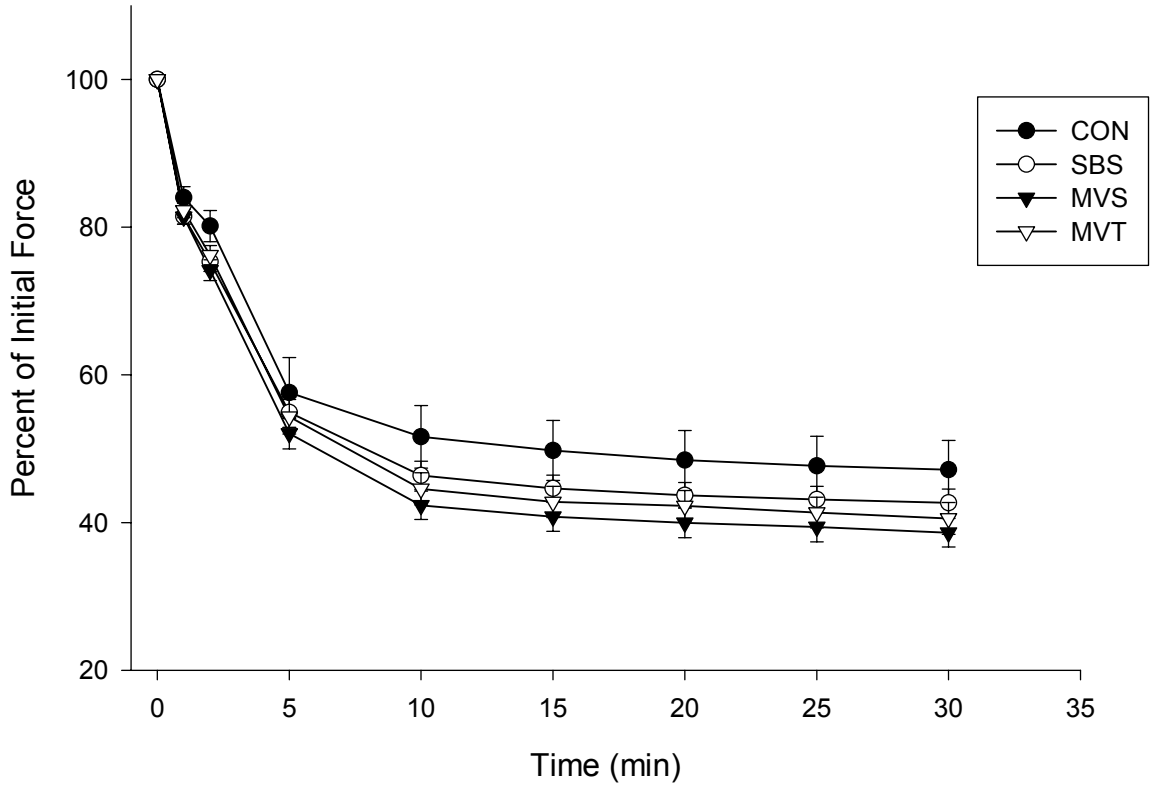

Figure 4-3. Percent of initial force maintained by *in vitro* diaphragm strips from control (CON), spontaneously breathing (SBS), mechanical ventilation (MVS), and mechanical ventilation animals receiving Trolox (MVT) after a 30-min fatigue protocol.

Values represent means ± SEM.
* Significantly different from CON group, $p<0.05$.
+ Significantly different from SBS group, $p<0.05$.
# Significantly different from MVT group, $p<0.05$.

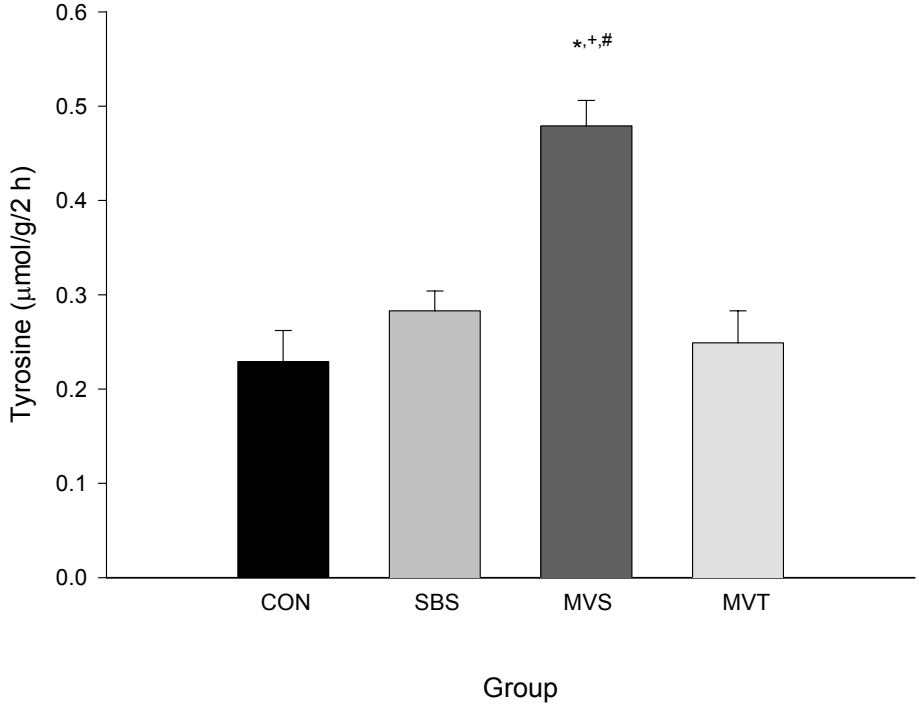

Figure 4-4. Total *in vitro* diaphragmatic protein degradation as measured by the rate of tyrosine release from control (CON), spontaneously breathing (SBS), mechanical ventilation (MVS), and mechanical ventilation animals receiving Trolox (MVT) during a 2-hour incubation.

Values represent means $\pm$ SEM.
* Significantly different from CON group, $p<0.05$.
+ Significantly different from SBS group, $p<0.05$.
# Significantly different from MVT group, $p<0.05$.

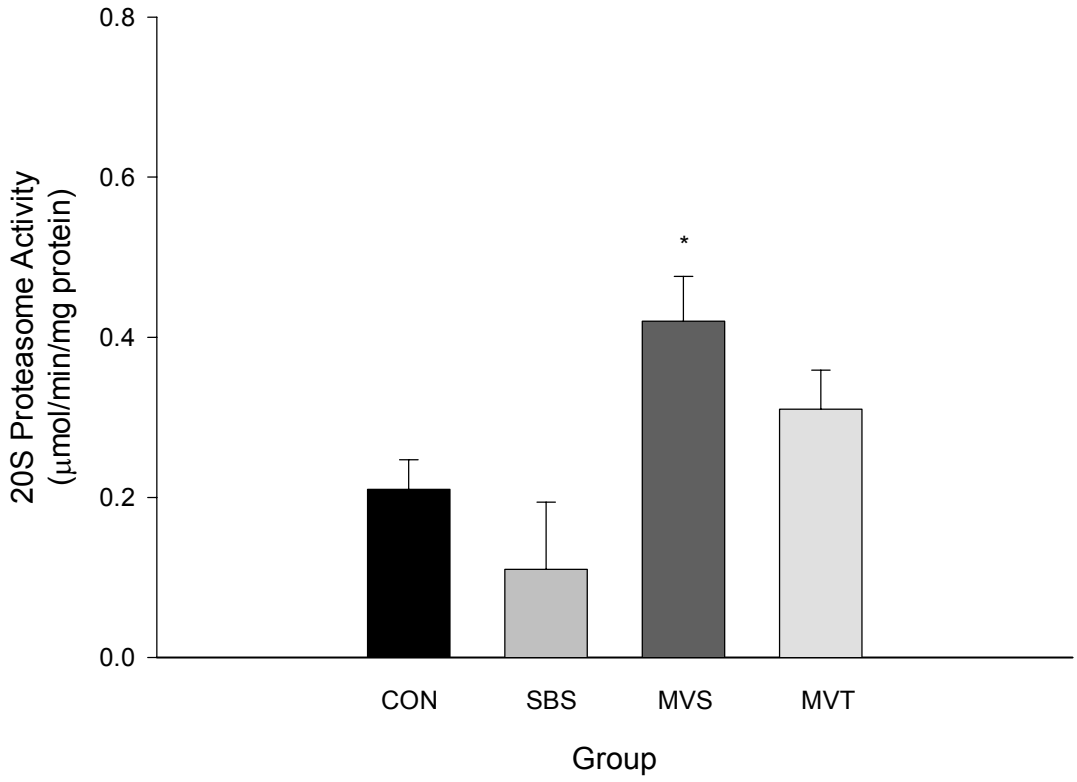

Figure 4-5. Chymotrypsin-like activity of the 20 S proteasome in diaphragm tissue from control (CON), spontaneously breathing (SBS), mechanical ventilation (MVS), and mechanical ventilation animals receiving Trolox (MVT).

Values represent means $\pm$ SEM.
* Significantly different from CON group, $p<0.05$.

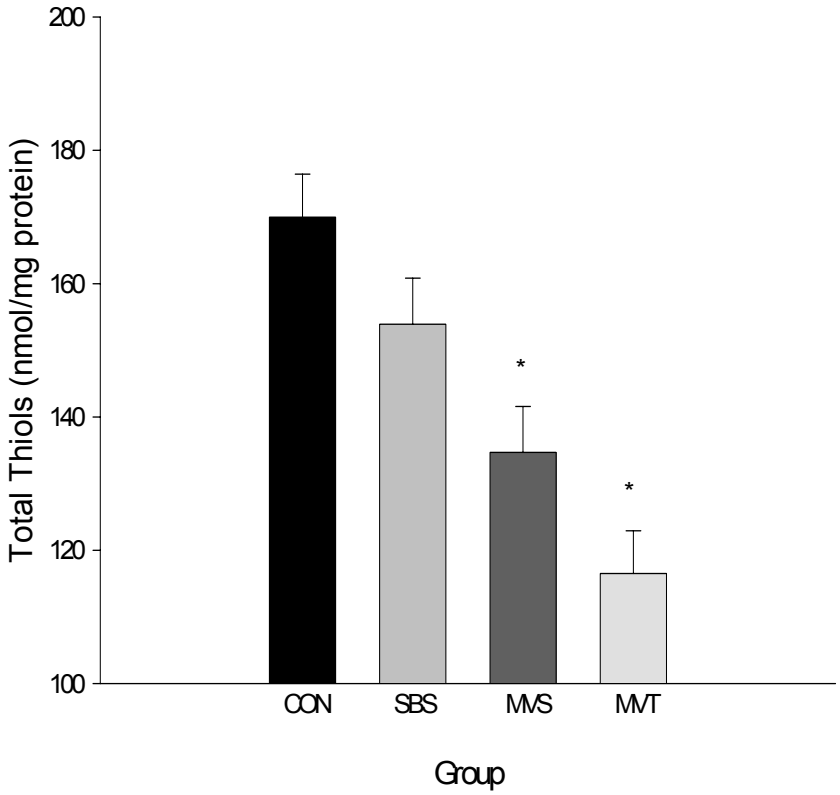

Figure 4-6. Total thiol concentration in diaphragm tissue from control (CON), spontaneously breathing (SBS), mechanical ventilation (MVS), and mechanical ventilation animals receiving Trolox (MVT).

Values represent means $\pm$ SEM.
* Significantly different from CON group, $p<0.05$.

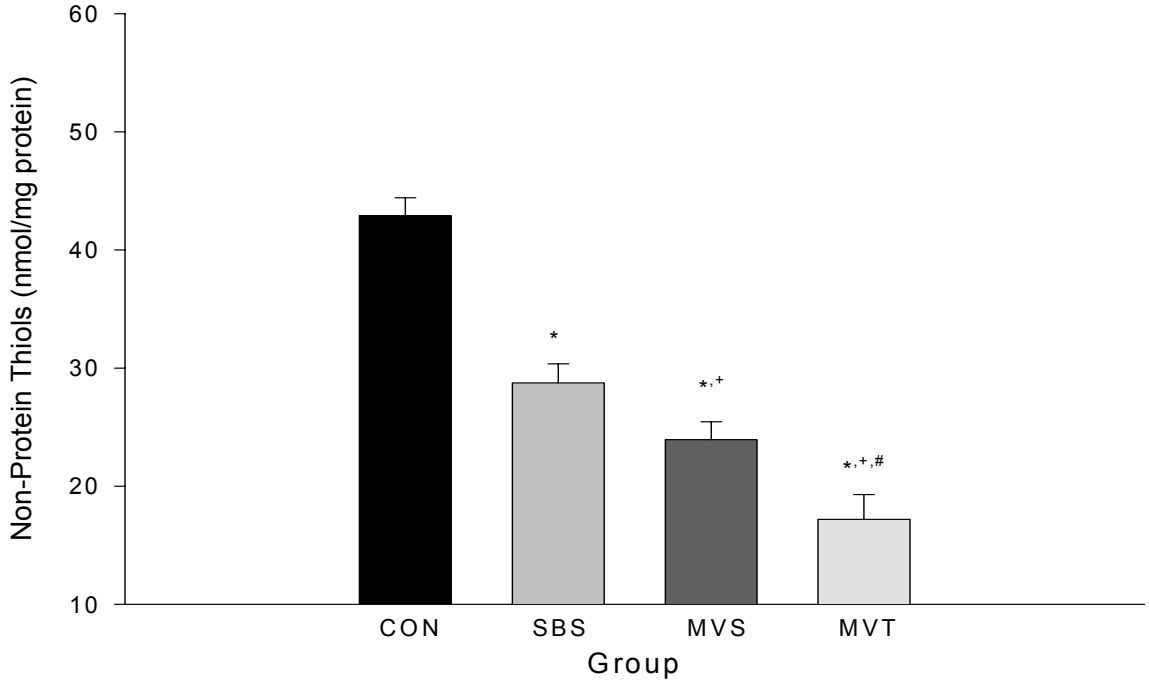

Figure 4-7. Non-protein thiol concentration in diaphragm tissue from control (CON), spontaneously breathing (SBS), mechanical ventilation (MVS), and mechanical ventilation animals receiving Trolox (MVT).

Values represent means ± SEM.
* Significantly different from CON group, $p<0.05$.
+ Significantly different from SBS group, $p<0.05$.
# Significantly different from MVS group, $p<0.05$.

CHAPTER 5
DISCUSSION

Trolox Attenuates Mechanical Ventilation-induced Contractile Dysfunction and Proteolysis in the Rat Diaphragm:Introduction

Weaning patients from a mechanical ventilator is a serious clinical issue. Mechanical ventilation (MV) is characteristically used in the clinical setting to maintain alveolar ventilation in patients who are incapable of ventilation on their own. As such, MV is an important life-preserving measure, but removing patients from the ventilator can be difficult in many cases. As many as 20% of patients experience difficulty in weaning from the ventilator (Lemaire 1993). Weaning procedures account for more than 40% of total MV time in patients who have difficulty weaning (Esteban 1994), suggesting that this is a serious clinical issue.

Our laboratory has reported that MV-induced diaphragmatic dysfunction is intrinsic to the muscle and increases in magnitude with increasing time on the ventilator (Powers et al. 2002). However, the mechanism(s) behind MV-induced diaphragmatic atrophy and weakness remain unclear. Since oxidative stress has been linked to reduced-use atrophy (Kondo et al. 1993), and protease-mediated protein degradation in unloaded locomotor muscle (Taillandier et al. 1996), we examined markers of oxidative stress during MV. Eighteen hours of controlled MV elevated markers of oxidative stress such as protein carbonyls and 8-isoprostanes (Shanely et al. 2002). This is significant because oxidized proteins are more prone to proteolytic attack and degradation (Dean 1997, Nagasawa et al. 1997). Indeed, 18 hours of MV is associated with an increase in protein degradation

through increases in total calpain-like activity and 20S proteasome activity (Shanely et al. 2002). Therefore, it seems logical that diaphragmatic weakening during MV-induced unloading may be caused by oxidative damage leading to heightened proteolytic degradation.

The purpose of this study was to determine whether supplementation with Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) during 12 hours of controlled MV would attenuate diaphragmatic contractile dysfunction, reduce oxidative stress, and attenuate protein degradation. Trolox is a water-soluble vitamin E analog with antioxidant properties (Wu et al. 1990, Zeng et al. 1991, Walker et al. 1998). We hypothesized that Trolox would maintain redox balance within the muscle by functioning as an antioxidant, and thereby prevent oxidative stress, and subsequent proteolysis and contractile dysfunction.

Materials and Methods

Animals and Experimental Design

Female Sprague-Dawley rats (~250 g) were obtained from Harlan (Indianapolis, IN). They were maintained on a 12-hour light:dark photoperiod and fed rat chow and water *ad libitum* prior to initiation of experiments. Animals were randomly assigned to one of five groups: (1) Controls receiving acute anesthesia and no further intervention (CON, n=8) (2) 12 hours of mechanical ventilation (MVS, n=8), (3) 12 hours of MV and Trolox infusion (MVT, n=8), (4) 12 hours of anesthesia and spontaneous breathing (SBS, n=8), and (5) 12 hours of anesthesia and spontaneous breathing with Trolox infusion (SBT, n=3). All procedures were approved by the University of Florida Animal Care and Use Committee.

Control Animal Protocol

The control animals (CON) were free of intervention before removal of the diaphragm for measurements. These animals received an acute intraperitoneal injection of sodium pentobarbital (65 mg/kg body weight). When a surgical plane of anesthesia was reached, the diaphragms were removed for measurement of *in vitro* contractile properties and tyrosine release, and remaining muscle was weighed and frozen for biochemical assays.

Mechanical Ventilation Protocol

Animals in the mechanically ventilated groups (MVS and MVT) were given an intramuscular injection of glycopyrollate (0.04 mg/kg) to reduce respiratory secretions. Thirty minutes later, subjects were anesthetized with an intraperitoneal injection of sodium pentobarbital (65 mg/kg). Upon reaching a surgical plane of anesthesia (no ocular response, no hindlimb withdrawal response), they were tracheostomized by an experienced lab technician and mechanically ventilated with a volume-cycled ventilator (Harvard Apparatus). The tidal volume was established at ~1 ml/100 g body weight with a respiratory rate of 80 breaths/min. Positive end-expiratory pressure of 1 cm H_2O was used for all ventilated animals. Throughout the MV period, heart activity, blood pressure, and core temperature were monitored. A lead II ECG displayed electrical activity of the heart. A catheter was placed in the carotid artery for constant blood pressure readings and arterial blood sampling. Core temperature was monitored with a rectal thermometer and adjustments were made to maintain body temperature at 37 ± 1^{o}C with a re-circulating heating blanket.

A catheter was placed in the jugular vein for the infusion of sodium pentobarbital (~10 mg/kg/h) and Trolox (Fluka). Constant supervision was provided for the rats

throughout the MV period. This included expressing the bladder, removing airway mucus, providing enteral nutrition, monitoring anesthesia rate, rotating the animals, lubricating the eyes, and infusing saline to maintain hydration status. To reduce airway secretions, glycopyrollate (0.04 mg/kg) was injected intramuscularly every 2 hours.

In the MVT group, the jugular vein was cannulated first. A priming dose of Trolox (20 mg/kg) was infused over a 5 min period. Twenty min later, MV was started, along with the constant infusion of Trolox at a rate of 5 mg/kg/h.

At the end of the 12-hour experimental period, all rats were killed by injection of sodium pentobarbital (50 mg/kg) and the diaphragm was removed for immediate analyses of contractile function and protein degradation as described below. Remaining costal diaphragm tissue was dissected, weighed, frozen, and stored for biochemical analyses.

Spontaneous Breathing Protocol

Spontaneously breathing (SB) animals were anesthetized in the same manner as MV animals and received sham surgeries. These animals were included in the study to determine whether long-term anesthesia (sodium pentobarbital) impairs diaphragmatic contractile function. A tube was inserted into the trachea, and these animals were maintained on a surgical plane of anesthesia for the 12-hour period while continuing to breathe on their own. The carotid artery and jugular vein were cannulated, and sodium pentobarbital was infused for the 12 hours. However, these animals were not mechanically ventilated.

The SBT group received the same dose of Trolox as the MVT group (20 mg/kg priming dose, 5 mg/kg/h constant infusion) for the 12-hour experimental period. The same general care was provided for these animals as for MV animals.

Contractile Measurements

The entire diaphragm with the supporting ribs and central tendon was removed and placed in a dissecting chamber containing a Krebs-Hensleit solution aerated with 95% O_2-5% CO_2 gas. The entire crural diaphragm was removed and discarded. A strip was cut from the midcostal region including the central tendon and the rib. This was secured vertically in an organ bath maintained at 24^{o}C between two Plexiglass clamps. The muscle strip was placed between two platinum field electrodes connected to an isometric force transducer (model FT-03, Grass Instruments, Quincy, MA). This strip was mounted on a micrometer to allow for muscle length adjustment. A 15 min equilibration period in the bath preceded all data collection. During this time, the remaining diaphragm muscle was dissected and sectioned. All sections were weighed, frozen in liquid nitrogen, and stored at –80^{o}C for further analyses.

We determined optimal muscle length (L_o), the length that generates maximal twitch force, and used this length throughout the protocol. L_o was found by systematically adjusting the length of the muscle while stimulating it with single supramaximal (~150%) twitches and recording the force generated. L_o was measured using calipers.

The force-frequency relationship was studied by stimulating the muscle strips at 15, 30, 60, 100, 160, and 200 Hz (120 V). Each stimulus was applied for 500 ms, and adjacent stimulus trains were separated by 2 min of rest. Peak isometric tetanic tension was determined from these measurements.

Diaphragmatic fatigability was assessed by monitoring the decrease in force development over time. Each muscle strip was stimulated with unfused tetanic contractions (30 Hz, 250 ms) for 30 min. The duty cycle, or time of muscle contraction compared to muscle rest, was 12.5%. Tension was measured at 0, 1, 2, 5, 10, 15, 20, 25,

and 30 min. Fatigue resistance was assessed by the percentage of initial force maintained at the end of the 30 min protocol. After all contractile measurements were taken, the diaphragm strip was removed from the organ bath. The rib, central tendon, and excess fat and connective tissue were removed from the strip, which was then weighed. Forces generated were normalized to muscle strip cross-sectional area (CSA), calculated from strip weight and length at Lo.

Protein Degradation

To measure total *in vitro* protein degradation, the release of free tyrosine into the incubation media was assayed. The rationale for this technique is that tyrosine is neither synthesized nor degraded by skeletal muscle, making it an ideal marker of total muscle protein breakdown (Tischler et al. 1982). Two strips were cut from the midcostal diaphragm (~ 40 mg each). These strips were secured at resting length in separate baths containing Krebs-Ringer bicarbonate solution, which was supplemented with 5 mM glucose, insulin (1 unit/ml), 0.17 mM leucine, 0.10 mM isoleucine, and 0.20 mM valine to improve protein balance, and 5 mM cycloheximide to inhibit protein synthesis. Diaphragm strips were maintained at resting length by securing both ends to a solid plexiglass support. Muscle strips were suspended vertically in the organ bath. The medium was continuously gassed with 95% O_2-5% CO_2, and temperature was maintained at 37^{o}C with a recirculating water bath. After a 30-min pre-incubation period, the media was drained, and fresh media was quickly added for a 2-hour incubation. Rates of total protein breakdown were measured by assaying tyrosine release into the medium according to the spectrofluorometric method of Waalkes and Udenfriend (1957).

20 S Proteasome Activity

The *in vitro* chymotrypsin-like activity of the 20S proteasome was measured fluorometrically by following the release of free AMC from the synthetic substrate Suc-Leu-Leu-Val-Tyr-AMC (Affiniti Research) using techniques described by Stein et al. (1996). Briefly, ~50 mg of midcostal diaphragm tissue was homogenized in buffer containing 50 mM Tris base, 1 mM EDTA, 1 mM EGTA, 1 μM Pepstatin-A, 50 μM E-64, and 10% glycerol. After initial centrifugations, the supernatant was collected and centrifuged at 100,000 x g in an ultracentrifuge for 1 hour at 4oC. This supernatant fraction was used to measure protein content using the Bradford method (Bradford 1976), and to measure 20S proteasome activity as follows. Ten μg of protein was reacted with the synthetic peptide substrate for chymotrypsin-like activity (Suc-LLVY-AMC) in a reaction mixture containing 50mM Tris-HCl, 1 mM DTT, and 5 mM $MgCl_2$. One aliquot from each sample was incubated with an inhibitor of the chymotrypsin-like proteasomal activity, lactacystin (Boston Biochem), while the other was not incubated with the inhibitor. Samples were incubated for 30 min at 37^{o}C before the addition of substrate. The change in fluorescence was measured at an excitation wavelength of 380 nM and emission of 460 nM. The difference between the activities of the proteasome with and without inhibitor was used as the 20S proteasome activity.

Statistical Analysis

Comparisons between groups were made by a one-way ANOVA. Where significant differences were found, Tukey's HSD test was implemented *post hoc*. Independently, the effects of anesthesia were compared using a Student's t-test on SBS and CON group data. Significance was established *a priori* at $p<0.05$.

Results

Systemic and Biologic Responses to Treatment

The MV protocol did not significantly change body mass for any of the 5 groups (Table 4-1), indicating that our schedule of nutrition and rehydration was adequate. The ratio of total costal diaphragm mass to final body mass was not significantly different between the 5 groups ($p=0.501$).

There were no signs of infection in any animals, and only 1 MVS animal was eliminated from the study due to evidence of barotrauma to the lungs on post-mortem examination. Systolic blood pressure was maintained at 70-110 mmHg in all groups, and arterial pH, PO_2, and PCO_2 were maintained within physiological ranges for both MV groups. The SB animals were mildly hypoxic, hypercapnic, and acidotic, as expected due to the anesthesia. Body temperature was kept at 37 ± 1^oC during the 12 hour protocol.

Effects of Anesthesia on Diaphragm Contractile Properties

The maximal tetanic force was not different between the SBS and CON groups (25.09 ± 0.41 N/cm^2 vs. 25.33 ± 0.50 N/cm^2, respectively). Likewise, the force-frequency curves and fatigue data were similar between these two groups (Figures 4-1, 4-2, and 4-3), and contractile parameters did not differ (Table 4-3). Thus, 12 hours of sodium pentobarbital anesthesia did not affect *in vitro* contractile properties of the diaphragm.

Effects of Mechanical Ventilation on Contractile Properties

Twelve hours of controlled MV reduced maximal tetanic force production by ~17% (21.00 ± 0.71 N/cm^2 vs. 25.43 ± 0.50 N/cm^2 in CON animals). The force-frequency curve of the MVS group was shifted downward and to the right of the CON group (Figure 4-1). This indicates a reduction in force generation at all stimulation frequencies tested. The fatigue protocol produced curves of similar shape for all groups (Figure 4-2), but the

MVS group generated a significantly lower amount of force compared to CON, SBS, and MVT groups where indicated. When the fatigue data are expressed as percent of initial force (Figure 4-3), there are no significant differences between the 5 groups at any time point ($p= 0.230$).

One-half relaxation time (½ RT) of maximal twitch was significantly shorter in the MVS group compared to CON, while ½ time to peak tension (½ TPT), rate of force development, and rate of relaxation were not different for either maximal twitch or maximal tetanic forces (Table 4-3).

Effects of Trolox on Contractile Properties

Trolox supplementation during 12 hours of MV completely attenuated the loss of maximal force generation. The MVT group was not significantly different from CON at any stimulation frequency tested (Figure 4-1). Animals receiving Trolox during MV maintained a greater force generating ability following the fatigue protocol compared to the unsupplemented MVS group (Figure 4-2). Trolox during MV significantly prolonged the rate of relaxation of maximal twitch compared to CON, but did not affect other contractile parameters (Table 4-3).

Twelve hours of Trolox infusion without MV (i.e. the SBT group) greatly increased subject mortality rate. Only three SBT animals survived the treatment. Further, diaphragms from these animals showed impaired force production compared to CON. The force-frequency curve of the SBT group was shifted downward and to the right of the CON group and closely resembles the MVS group (data not shown). Trolox supplementation to SB's also significantly reduced ½ TPT of maximal tetanic forces compared to CON animals (Table 4-3).

Protein Degradation

Twelve hours of controlled MV significantly elevated total *in vitro* protein degradation (+105%), as measured by the release of free tyrosine, compared to CON (Figure 4-4). However, protein degradation of the MVT group (16% increase compared to CON) was not significantly different from CON (p=0.797). There were no significant differences in protein degradation between CON and SBS groups (p=0.351).

20S Proteasome Activity

The chymotrypsin-like activity of the 20S proteasome was significantly increased in the MVS group compared to the CON group (+76%) (Figure 4-5). Trolox attenuated the MV-induced increase in proteasome activity (+26% compared to CON, p=0.647).

Discussion

Major Findings

The major findings of this study are: 1) Trolox supplementation during 12 hours of controlled MV attenuates diaphragmatic contractile dysfunction and whole muscle proteolysis; 2) 12 hours of anesthesia and spontaneous breathing do not affect contractile function or protein degradation within the diaphragm; 3) Proteolysis is elevated during 12 hours of MV in part due to increased chymotrypsin-like activity of the 20S proteasome, and 4) Trolox supplementation during normal spontaneous breathing shifts redox balance to a reductive state which actually impairs diaphragmatic function.

MV and Diaphragmatic Dysfunction

These data support our previous studies (Powers et al. 2002, Shanely et al. 2003) that show a reduction in maximal force production with prolonged MV. These data agree with other MV studies with rats (Le Bourdelles et al. 1994), baboons (Anzueto et al. 1997), piglets (Radell et al 2002), and rabbits (Sassoon et al. 2002, Capdevila et al.

2003). In the present study, maximal tetanic tension was decreased ~17% with 12 hours of controlled MV. Le Bourdelles et al. (1994) demonstrated that 48 hours of MV significantly reduced diaphragmatic force without altering protein concentrations or enzyme activities. Anzueto et al. (1997) found a decrease in transdiaphragmatic pressure and diaphragmatic endurance after 11 days of MV in baboons. However, the use of long-lasting neuromuscular blockers in this study may have affected diaphragm responses. We have shown that the degree of diaphragmatic dysfunction is proportional to the length of time of MV (Powers et al. 2002).

Radell and colleagues (2002) demonstrated that diaphragmatic dysfunction induced by 5 days of MV in a piglet model is not associated with alterations in nerve function or neuromuscular transmission. With respect to muscle fiber composition, Capdevila et al. (2003) found significant atrophy of type IIa and IIb fibers, with no changes in type I fibers, in the rabbit diaphragm ventilated for 51 hours. In the rat, 4 days of MV caused a decrease in the percentage of type I fibers, and an increase in hybrid fibers co-expressing type I and II MHC (Yang et al. 2002). Also, Shanely and colleagues (2002) found atrophy of all fiber types that was greatest in type II fibers after just 18 hours of MV. Finally, significant myofibril damage in the diaphragm is evident after MV (Sassoon et al. 2002).Together, these alterations in diaphragmatic structure may lead to the weakness characteristic of a mechanically ventilated diaphragm muscle.

In the present study, Trolox completely attenuated the decrease in maximal specific tension that occurs during MV. These animals were not different from CON or SBS animals with respect to maximal tetanic tension, maximal twitch tension, force-frequency relationships, and fatigue data.

Oxidative Stress and Trolox

Unloaded skeletal muscle is susceptible to oxidative stress during periods of disuse (Kondo et al. 1993a, 1993b). During MV, the diaphragm muscle is both unloaded and passively shortened (Racz et al. 2003). These stimuli are likely to stimulate the unloading-induced atrophy typical of prolonged MV. Previous studies from our laboratory have measured an increase in protein oxidation and lipid peroxidation with 18 hours of MV (Shanely et al. 2002), and as early as 6 hours of MV (Zergeroglu et al. 2003). In the present study, we measured a decrease in total and non-protein thiols with 12 hours of MV consistent with oxidative stress (data not shown). However, Trolox did not prevent the loss of thiol groups during MV. It may be that specific proteins, such as myosin, are preferentially spared from oxidation while others are not. If true, we were not able to detect these proteins in crude homogenate. However, we postulate that Trolox is functioning as an antioxidant to prevent oxidative stress. Several studies have shown that Trolox reduces oxidative stress induced by cumene hydroperoxide (Persoon-Rothert et al. 1990), methylmercury intoxication (Usuki et al. 2001), and other oxyradicals (Wu et al. 1990, Wu et al. 1991, Zeng et al. 1991, Walker et al. 1998). Wu and colleagues (1990) have even demonstrated antioxidant actions of Trolox in three human cell types exposed to oxyradicals. While we were unable to clearly demonstrate the prevention of oxidative stress with Trolox, it is likely that Trolox is acting as an antioxidant to scavenge reactive oxygen species produced in the diaphragm during 12 hours of controlled MV.

MV and Proteolysis

Twelve hours of controlled MV significantly increased (+105%) the release of tyrosine from *in vitro* diaphragm strips. This agrees with a previous study from our laboratory reporting increased tyrosine release after 18 hours of MV (Shanely et al.

2002). Tyrosine release is frequently used as an indicator of total protein degradation (Lowell et al. 1986) since tyrosine is neither synthesized nor degraded by skeletal muscle. In our previous 18-hour MV study, we determined that total calpain-like and 20S proteasome activities were elevated, indicating a contribution of these two proteolytic pathways to diaphragmatic proteolysis during MV (Shanely et al. 2002).

Importantly, Trolox attenuated the increase in total protein degradation induced by MV (Figure 4-4). Likewise, the chymotrypsin-like activity of the 20S proteasome was elevated during MV, but this increase was prevented with Trolox (Figure 4-5). Many studies indicate that the proteasome is responsible for ~70-80% of the increased cellular protein degradation following an oxidative stress (Grune et al. 1995, Grune et al. 1996, Grune and Davies 1997). More specifically, it appears the 20S proteasome is responsible for the degradation of oxidized proteins since the 26S proteasome is inhibited/inactivated by oxidative stress (Reinheckel et al. 1998). Recognition of exposed hydrophobic patches is the proposed mechanism by which the proteasome selectively degrades oxidatively modified proteins (Grune et al. 1997). Oxidative damage to a protein leads to partial unfolding and exposure of normally shielded internal hydrophobic patches that are recognized by the proteasome, which catalyzes the degradation of that protein.

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

Critique of Experimental Model

It was necessary to use an animal model due to the invasive nature of this study. The rat was selected due to the similarities in anatomy and function of the rat and human diaphragms. Also, controlled MV, which is not as common clinically as pressure assist MV, was used because of the rapid onset of diaphragmatic atrophy characteristic of controlled MV.

Prolonged anesthesia is known to lead to hypoxia, hypercapnia, and mild acidosis due to the reduced ventilatory drive in an anesthetized animal (Powers et al. 2002). While these effects could theoretically affect *in vitro* diaphragm function, our data demonstrate that sodium pentobarbital anesthesia does not impact skeletal muscle function *in vitro*. A comparison of the CON and SBS data indicate no significant differences in force generation, total proteolysis, or proteasome activity. Thus, the diaphragmatic dysfunction and proteolysis induced by MV were not caused by prolonged sodium pentobarbital use.

We attempted to include a group to control for Trolox during spontaneous breathing (SBT group). However, only 3 animals from this group survived the entire 12 hours, and these were not included in statistical analyses. It is not surprising that Trolox was harmful in these animals that were not exposed to oxidants because Trolox is a strong reductant. We hypothesize that Trolox shifted the redox balance to a reductive state in the spontaneously breathing animals that impaired diaphragmatic function.

Conclusions

Our results support earlier conclusions that short-term controlled MV leads to diaphragmatic contractile dysfunction and increased protein degradation. Our data clearly demonstrate that an antioxidant, Trolox, effectively prevented contractile impairments and proteolysis during MV. Oxidative damage and atrophy are implicated in MV-induced

contractile deficits. Oxidative damage to proteins during MV likely increases proteolytic degradation, which would contribute to diaphragmatic weakness. Trolox effectively spares the unloaded diaphragm from contractile dysfunction, oxidative stress, and protein degradation during 12 hours of controlled MV. However, it is not warranted during normal spontaneous breathing, and can actually cause contractile dysfunction under such conditions.

Future studies aiming to prevent force losses, oxidative stress, and protein degradation during MV might examine another vehicle and route of Trolox administration. In addition, other antioxidants need to be tested. The use of an antioxidant such as Trolox may prove beneficial in the clinical setting where weaning difficulties are encountered due to diaphragmatic atrophy and weakness. This is a serious clinical issue that warrants further investigation.

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BIOGRAPHICAL SKETCH

Jenna Betters was born in New Smyrna Beach, Florida, and graduated salutatorian of her high school class in 1997. She received her bachelor's degree in biology from the University of North Florida, Jacksonville, Florida, in May of 2001, where she was a member of Phi Kappa Phi Honor Society, University Scholar's Honor Society, and Golden Key International Honor Society. She began a master's program in exercise physiology at the University of Florida, Gainesville, Florida. She has worked for two years as a fitness trainer, and three years as a research assistant in the Molecular Physiology Laboratory within the Center for Exercise Science. Jenna has been accepted into the doctoral program and will pursue a Ph.D. in exercise physiology from the University of Florida.