MECHANISMS OF MECHANICAL VENTILATION-INDUCED OXIDATIVE STRESS IN THE DIAPHRAGM

By

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This dissertation is dedicated to my parents, Bill and Carol.

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Mechanical ventilation (MV) is associated with oxidative stress and contractile dysfunction in the diaphragm. The pathways responsible for the production of oxidants in the diaphragm during MV remain unknown. To address this issue, these experiments tested the following hypotheses: 1) NADPH oxidase activity is increased during MV and contributes to the oxidative stress and contractile dysfunction of the diaphragm; and 2) diaphragmatic nitric oxide synthase (NOS) levels are elevated in the diaphragm during MV and contribute to nitration of proteins in the diaphragm. To test these postulates, rats were mechanically ventilated for 18 hours with a subset of animals receiving the NADPH oxidase inhibitor, apocynin (4 mg/kg body weight). Diaphragmatic NADPH oxidase and NOS activities were measured along with protein levels of all three NOS isoforms. Further, 3-nitrotyrosine levels in the diaphragm were measured as an index of protein nitration. Compared to control, MV resulted in diaphragmatic oxidative stress and a significant decrease (-10%) in the maximal specific force of the diaphragm. MV did not

increase diaphragmatic NADPH oxidase activity above control. Nonetheless, the administration of apocynin attenuated MV-induced contractile dysfunction. Interestingly, treatment with apocynin did not diminish diaphragmatic NADPH oxidase activity but protected the diaphragm against MV-induced oxidative stress. Moreover, MV did not promote an increase in diaphragmatic protein levels of eNOS, nNOS, or iNOS or NOS activity. Consistent with these findings, MV did not elevate diaphragmatic protein levels of 3-nitrotyrosine in any region of the diaphragm including the insoluble, cytosolic, mitochondrial, and membrane protein fractions. Therefore, we conclude that MV-induced oxidative stress in the diaphragm is not due to increases in NADPH oxidase activity or increased NO production by NOS. Moreover, our results suggest that apocynin attenuates the MV-induced diaphragm contractile dysfunction and oxidative stress via its antioxidant properties, not through the inhibition of NADPH oxidase.

CHAPTER 1
INTRODUCTION

Mechanical ventilation (MV) is used to maintain adequate alveolar ventilation in patients incapable of doing so on their own. The removal of patients from the ventilator is termed weaning. Difficulty in weaning is often defined as a weaning procedure requiring more than 48 hours to permanently remove patients from the ventilator (1). Importantly, difficulties in weaning occur in approximately 25% of patients utilizing MV (1). Clinically, this is significant because weaning accounts for greater than 40% of the total time on the ventilator (2).

There are two major modes of MV: pressure-assist and controlled. As the name implies, pressure-assist MV assists the patients' inspiratory efforts, while during controlled MV, the ventilator delivers all of these breaths, therefore rendering the inspiratory muscles inactive. Pressure-assist MV is commonly utilized in adult patients suffering from acute respiratory failure whereas controlled MV is utilized in instances where patients suffer from a spinal cord injury or during surgery (3). Furthermore, controlled MV is commonly used in pediatric situations (3). Given that controlled MV completely inactivates the respiratory muscles, as revealed by the absence of EMG activity in the diaphragm during MV, it is likely that diaphragmatic atrophy and dysfunction associated with MV occur rapidly using this mode of MV (3-5).

To study the effects of MV on the diaphragm, an animal model must be utilized because of the invasive nature of removing diaphragm muscle samples. Thus far, four animal models have been utilized including the baboon, pig, rat, and rabbit. Of these

models, the rat has been utilized in our laboratory due to similarities to the human diaphragm in fiber type, biochemical properties, anatomical features, and physiological function (6-8).

Previously, our laboratory has demonstrated a decrement in the specific force of the diaphragm following as little as 12 hours of MV (4). Further, this deficit is exacerbated to a 60% loss in force following 48 hours of MV (9). During MV, our laboratory (10) and others (9, 11-14) have noted an increase in diaphragmatic atrophy; however, since the specific force of the diaphragm is normalized per cross sectional area, the decline in muscle force production is not due to atrophy alone.

One proposed mechanism by which diaphragmatic dysfunction may occur during MV is via oxidative stress-induced injury to the diaphragm. Our group has found an increase in protein oxidation and lipid peroxidation following as little as six hours of MV (10, 15). These oxidized proteins can be targeted by the proteasome proteolytic system where they are degraded by the 20S proteasome, thereby accelerating muscle atrophy (16, 17). Further, an increase in oxidative stress can damage proteins involved in excitation-contraction coupling, reducing muscle force production (18-20). The physiological significance of MV-induced oxidant stress in the diaphragm has been confirmed by recent experiments demonstrating that infusion of the antioxidant, Trolox, prevents MV-induced contractile dysfunction in the diaphragm (21).

Various sources of reactive oxygen species (ROS) exist in skeletal muscle including the calcium-activated enzyme, NADPH oxidase. This source of ROS is responsible for the one electron reduction of oxygen into superoxide using NADPH or NADH as the electron donor (22). The inhibition of NADPH oxidase derived superoxide

can be achieved with the addition of apocynin (4-hydroxy-methoxyacetophenone; acetovanillone). Apocynin most likely reduces superoxide formation by blocking sulfhydryl groups and inhibiting NADPH oxidase enzyme assembly (23). Previous investigators have successfully used apocynin *in vitro* and *in vivo* to inhibit NADPH oxidase activity in skeletal muscle (22, 24). NADPH oxidase has been well characterized in the mammalian diaphragm and remains as a possible source for oxidant production in inactive skeletal muscle.

Also, nitric oxide (NO) has been reported to be produced in immobilized locomotor muscle and therefore could also contribute to oxidative injury in the diaphragm during MV (25). NO is produced by calcium-activated cellular enzyme, nitric oxide synthase (NOS). NO can react with superoxide to form the highly reactive peroxynitrite molecule that can nitrosylate proteins in skeletal muscle.

Given that MV-induced oxidant stress is associated with diaphragmatic contractile dysfunction, determining which oxidant producing pathways are responsible for the generation of reactive oxygen species is important. Based on preliminary experiments, we formed the working hypothesis that MV results in an increase in oxidant production in the diaphragm via increased NADPH oxidase activity and an increase in nitric oxide synthase (NOS). More specifically, the current experiments are designed to test two hypotheses: 1) NADPH oxidase activity is increased in the diaphragm during MV and contributes to diaphragmatic oxidative injury and contractile dysfunction; and 2) diaphragmatic NOS levels are elevated in the diaphragm during MV and contributes to the nitration of proteins in the diaphragm.

CHAPTER 2
LITERATURE REVIEW

Mechanical ventilation (MV) is utilized to maintain adequate alveolar ventilation in patients incapable of doing so on their own. The process of removing patients from the ventilator is termed weaning, and problems in weaning occur in approximately 25% of patients exposed to MV for two or more days (1). One of the proposed mechanisms by which weaning difficulties occur is due to impairments in diaphragmatic strength and endurance (4, 9, 11-13, 26-29). Importantly, recent experiments in our laboratory suggest that MV-induced oxidative stress contributes to both diaphragmatic atrophy and contractile dysfunction (10, 15). Indeed, it is well established that an increase in oxidative modification of proteins and lipids in the diaphragm can promote skeletal muscle atrophy and dysfunction (10, 15). This review will outline our current understanding of MV-induced diaphragm dysfunction and will also provide a brief overview of oxidant producing pathways that could be responsible for MV-induced oxidative damage in the diaphragm.

Mechanical Ventilation-Induced Diaphragmatic Dysfunction

Muscle Force

Utilizing a variety of different animal models, diaphragmatic dysfunction has been evaluated following MV (4, 9-12, 14, 15, 21, 26-28, 30-35). These experiments reveal that the *in vivo* transdiaphragmatic pressure of baboons, piglets, and rabbits is significantly reduced following MV (11, 27, 28). For example, diaphragmatic force decrements have been shown to occur as early as one day in rabbits and three days in

piglets; these force decrements are exacerbated with time on the ventilator (27, 28). Further, there is a 40-50% decline in the pressure-generating capacity of the diaphragm after 3 days in rabbits, 5 days in piglets, and 11 days in baboons (11, 27, 28). Also, it has been shown that following a prolonged period of MV, animals cannot sustain diaphragmatic force under an inspiratory resistive load, indicative of an endurance decrement of the diaphragm (11). Importantly, these impairments in diaphragmatic function are not attributable to changes in lung volume and abdominal compliance or the function of the phrenic nerve and neuromuscular junction (11, 27).

In addition to the aforementioned *in vivo* experiments, *in vitro* preparations have also been utilized to determine the force of isolated rat and rabbit diaphragm strips removed from animals following varying periods of MV. A 30-50% reduction in the maximal isometric specific force of the diaphragm occurs after one to three days of MV (4, 9, 12, 13, 28) with as little as 12 hours of MV resulting in an approximately 20% decrement in force (4, 26). The decrements in force cannot be attributed to atrophy alone due to the fact that the force of the diaphragm in these studies has been normalized to the cross-sectional area. Furthermore, the diaphragm strip is set at the optimal length; therefore, the force decrement cannot be due to altered muscle operating length. Finally, anesthetic agents and neuromuscular blockers are not the cause of the diaphragmatic dysfunction following MV. This has been experimentally demonstrated by omitting neuromuscular blockers from several studies and the anesthetic agent has been controlled by utilizing a spontaneously breathing group of animals under anesthesia, but not undergoing MV (36).

The endurance of the diaphragm following MV has also been assessed *in vitro* (13, 28, 32, 33). Conclusions on the fatigue resistance of these diaphragm strips are inconsistence with studies reporting an increase (32), decrease (33), or no change in the endurance of the diaphragm following MV (13, 28). Therefore, it is not clear what the effects of MV are on the endurance of the diaphragm.

In humans, it is much more difficult to study the effects of MV on diaphragmatic function. Confounding factors such as various disease states, drugs, and modes of ventilation make it difficult to determine the cause of respiratory muscle dysfunction. Further, assessment of diaphragm function in humans is complicated given that the obtainment of diaphragm samples is invasive. However, histopathologic analysis of diaphragms from 13 neonates ventilated 12 days or more suggests that diaphragm fiber atrophy occurs (37). Moreover, 33 patients with various diseases exposed to two or more days of MV exhibited a 50% decrement in the twitch transdiaphragmatic pressure from supramaximal magnetic stimulation of the phrenic nerve when compared to normal subjects (29). Therefore, although data from human studies are limited, the current results are consistent with animal experiments indicating that prolonged MV results in diaphragmatic atrophy and contractile dysfunction.

MV-Induced Atrophy

MV-induced diaphragm atrophy has been reported in almost all animal experiments (9, 11-14). MV-induced diaphragmatic atrophy occurs more rapidly (e.g., 18 hours) than disuse atrophy of peripheral skeletal muscles (9, 10, 12). Disuse muscle atrophy can occur due to a decrease in protein synthesis (38), an increase in protein degradation (39), or a combination of both. Our laboratory has shown a decrease in protein synthesis during as little as six hours of MV (34). Moreover, mRNA for insulin-like growth factor

(IGF-1) and type I and IIx myosin heavy chain are depressed after 18 to 24 hours of MV (13, 34). Importantly, our group has reported an increase in proteolysis after 18 hours of MV (10). Based upon the relatively long half-life of many skeletal muscle proteins, it is feasible that the rapid onset of diaphragmatic atrophy is primarily due to a rapid onset of proteolysis.

There are three primary pathways involved in proteolysis in skeletal muscles: 1) lysosomal proteases (cathepsins), 2) calcium activated neutral proteases (calpain), and 3) the proteasome. In regard to protease activation in the diaphragm during MV, Shanely et al. (10) have reported a greater than twofold increase in diaphragmatic calpain activity and approximately a fivefold increase in 20S proteasome activity following 18 hours of MV. Calpains are responsible for the release of myofilaments from the sarcomere and allow them to be degraded by the proteasome (40). The proteasome system consists of the ATP-dependent 26S proteasome, which requires the ubiquitination of proteins prior to degradation and the 20S proteasome that degrades proteins oxidized by reactive oxygen species (ROS) without the need for ATP or ubiquitin (36). An increase in calpain-like activity and 20S proteasome activity has been reported in the diaphragm following MV (10).

Structural Injury

During MV, alterations in the structure of the diaphragm such as disrupted myofibrils (14, 28), an increased number of lipid vacuoles in the sarcoplasm and abnormally small mitochondria with focal membrane disruptions have been reported in rabbits (14). Investigation on the external intercostals revealed similar findings (14). In contrast, inactive hindlimb muscles removed from animals undergoing MV do not exhibit these modifications (28). Myofibril disruption is physiologically significant because an

increase in abnormal myofibrils has been shown to be significantly correlated with decrements in diaphragmatic force output (28). A definitive explanation for MV-induced myofilament disruption does not currently exist. However, at least two possible explanations exist. First, the calcium-activated protease calpain could be releasing the myofilaments from the sarcomere (10). Secondly, periods of spontaneous breathing could occur during MV that would reload the diaphragm. Reloading of hindlimb muscles following prolonged periods of disuse has been associated with an increased susceptibility to muscle fiber injury (41). Nonetheless, we have demonstrated that two hours of reloading the diaphragm following 24 hours of MV does not exacerbate MV induced contractile dysfunction or cause membrane damage (35). Therefore, during periods of 24 hours of MV or less, spontaneous breathing does not appear to contribute to the structural damage that occurs in the diaphragm.

Oxidative Stress

It is well established that when cellular oxidant production exceeds the capacity of intracellular antioxidants to scavenge these oxidants, oxidative damage to cellular biomolecules occurs. In this regard, our research team has reported an increase in protein oxidation and lipid peroxidation in the diaphragm following various periods of controlled MV (10, 15). Interestingly, MV-induced oxidative injury occurs in the diaphragm within as few as six hours after the onset of MV (15). In this study, oxidized proteins were separated using SDS-PAGE and it was determined that the contractile proteins, actin and myosin, are oxidized in the diaphragm during prolonged MV (15).

At present, the pathways responsible for MV-induced oxidative stress in the diaphragm are unknown. Major oxidant producing pathways in cells include the electron

transport chain in the mitochondria, reactive iron, xanthine oxidase, NADPH oxidase, and nitric oxide synthase. A brief overview of each of these pathways follows.

Mitochondria

Mitochondria primarily function to produce ATP; however, they have also been shown to produce superoxide radicals (42). The major site of superoxide production within the mitochondria is the electron transport chain (42). Early components of the electron transport chain can leak electrons directly onto oxygen while most of the electrons are transferred to the next component of the chain. It is this leakage of electrons that generates superoxide (42).

During resting conditions (state four respiration), there is a high degree of reduction of the electron carriers and a limited supply of ADP as compared to periods of increased muscle contractile activity (state three respiration) (43). Therefore, during state four respiration, there is a greater proportional amount of superoxide production and the production of superoxide has been estimated to account for more than two percent of the oxygen consumed (43).

Moreover, with increasing concentrations of oxygen, there is an increase in electron leakage and, therefore, superoxide production (44). However, at physiological concentrations of oxygen, it has been estimated that only one to three percent of the oxygen reduced in the mitochondria forms superoxide (44). The low rate of leakage is most likely due to low oxygen concentrations within the mitochondria and the facilitation of electron flow by electron carrier complexes. At present, it is unknown if the mitochondria are a significant source of ROS in the diaphragm during MV.

Reactive iron

The highly reactive hydroxyl radical can be produced from the reaction of superoxide with hydrogen peroxide, but without reactive metals present, this reaction is too slow to be of physiological significance (45). In contrast, in the presence of a metal catalyst like iron or copper, this reaction, termed the Haber-Weiss reaction, will proceed much more rapidly (45).

During disuse atrophy, muscle fiber volume decreases rapidly and cell structure changes greatly which can disturb the balance of metals (46, 47). An increase in iron has been noted throughout 12 days of muscle disuse atrophy as revealed by an increase in the microsomal fraction of iron (46, 48). Further, an increase in a 54 kDa iron binding protein in the sarcoplasmic reticulum has been observed as early as four days after the initiation of skeletal muscle immobilization (46). Finally, when animals were treated with the iron chelator, deferoxamine, during skeletal muscle immobilization, there was a decrease in disuse-induced lipid peroxidation and oxidized glutathione (GSSG) in the immobilized muscles (46). However, when the deferoxamine was saturated with iron, markers of oxidative stress did not change.

One of the potential sources of free iron is heme oxygenase (HO). HO degrades heme into iron, carbon monoxide, and biliverdin (49). Of the two isoforms of HO (e.g., HO-1 and HO-2), HO-1 is the inducible isoform. Increases in oxidant stress have been shown to induce HO-1 and HO can serve as a pro or antioxidant (49). By degrading heme and releasing iron, HO acts as a pro-oxidant. In contrast, HO can also act as an antioxidant. For example, HO produces biliverdin that can be converted into bilirubin, and both compounds are antioxidants. Further, HO produces the iron binding protein ferritin that acts as an antioxidant by binding free iron and therefore preventing the iron-

mediated catalyzed formation of the hydroxyl radical (49). At present, it is unclear if HO acts as a pro-oxidant or an antioxidant in the diaphragm and this remains as an important area for future research.

Xanthine oxidase

Xanthine oxidase (XO) is present in the cytoplasm of skeletal muscle and exists in two forms: NAD-dependent (type D) and superoxide-producing (type O) (50). Both types have been shown to increase during 12 days of disuse muscle atrophy with increases in type O being greater (2.3-fold higher). Further, the substrates of XO, xanthine and hypoxanthine, are increased during muscle disuse as well as their product (i.e., urate). The larger increase in type O XO increased the ratio of type O XO to total XO which is indicative of the conversion of type D to type O XO (50). It is known that this conversion is catalyzed by calcium activated neutral proteases (calpain) (51). This is consistent with data indicating an increase in intracellular calcium during disuse atrophy (48, 52). Nonetheless, it is unclear if XO-induced production of oxidants is operative in the diaphragm during prolonged MV.

NADPH oxidase

NADPH oxidase is a membrane-associated enzyme that produces superoxide via a one-electron reduction of oxygen using NADPH or NADH as the electron donor. The enzyme exists in phagocytes (53) and nonphagocytes. Several differences exist between NADPH oxidases found in phagocytic and nonphagocytic cells including enzyme orientation, direction of superoxide production, subunit structure, and substrate preference (54). The phagocytic NADPH oxidase consists of five subunits including a plasma membrane-spanning cytochrome b558 that is composed of the p22phox and gp91phox subunits. Also, there are three subunits located in the cytosol including the

p47phox, p67phox, and p40phox. The cytosolic subunits are not associated with the membrane bound cytochrome until activation. Upon activation, the utilization of intracellular NADPH or NADH causes the transfer of electrons to oxygen in the extracellular space, producing superoxide outside of the cell (22).

Recently, it was discovered by Javesghani et al. (22) that the p22phox, gp91phox, p67phox, and p47phox subunits' mRNA and protein were located in skeletal muscle while p40phox was only in the blood vessels. Furthermore, this group discovered that the four subunits are constitutively expressed in the membrane, unlike the phagocytic NADPH oxidase. Importantly, they demonstrated that NADPH oxidase was capable of significant level of superoxide production in skeletal muscle. Given that NADPH oxidase can be activated by increases in intracellular calcium levels, we postulate that NADPH oxidase is a possible source of oxidant production in the diaphragm during prolonged MV.Nitric oxide (NO)

Nitric oxide (NO)

The free radical, nitric oxide (NO), is produced by the enzyme NO synthase (NOS). There are three isoforms of NOS: 1) type I or neuronal (nNOS), 2) type II or inducible (iNOS), and 3) type III or endothelial (eNOS). All forms of NOS produce NO from L-arginine and require oxygen and NADPH as substrates while citrulline is a byproduct (55). The cofactors involved in this reaction include FAD, FMN, tetrahydrobiopterin, heme, and calmodulin. The binding of NOS and calmodulin is calcium dependent with only the constitutive isoforms (nNOS and eNOS) being calcium sensitive (55). The calcium sensitive binding of calmodulin is the primary regulator of the production of NO via the constitutive isoforms of NOS; however, iNOS is tightly

bound to calmodulin and not calcium regulated. iNOS is primarily regulated at the transcriptional level and is upregulated due to an inflammatory challenge (55).

Targets of NO can be generalized into three main categories. First, NO can react with oxygen and superoxide to form low molecular weight NO derivatives such as peroxynitrite. These molecules maintain redox activity and can participate in the transfer of electrons (56). The NO derivative, peroxynitrite, is one of the most reactive free radicals involved in oxidative damage within skeletal muscle (57). Second, derivatives of NO can react with transition metals such as iron to produce NO-metal adducts. This is a mechanism by which NO modulates metalloprotein function. Third, one of the main targets of NO is reduced thiols. NO can cause the S-nitrosylation of protein thiols, which is reversible (55). The formation of 3-nitrotyrosine is one of the most commonly studied covalent modifications of proteins by NO (58).

Summary

Previous work has demonstrated that control MV renders the diaphragm completely inactive. During this period of disuse, diaphragmatic force decrements occur within as little as 12 hours of MV. Further, diaphragmatic atrophy occurs but cannot account for all of the dysfunction due to the fact that the force of the diaphragm is normalized per cross sectional area.

MV has also been associated with an increase in oxidative stress in the diaphragm. Specifically, following as few as six hours of MV, increased lipid peroxidation and protein oxidation occurs within the diaphragm. This is physiologically significant because MV-induced oxidative stress can accelerate diaphragmatic proteolysis and contractile dysfunction. Numerous biochemical pathways could be involved in the increase in oxidants that cause damage to the diaphragm including the mitochondria, free

(reactive) iron, xanthine oxidase, NADPH oxidase, and NO. While all of these pathways are potential contributors to the increase in oxidative stress that occurs during MV, the primary aim of this investigation is to determine if the NADPH oxidase pathway and NO pathway are involved in MV-induced oxidative injury in the diaphragm. Our long-term objective is to determine which ROS pathways are involved in oxidant production in the diaphragm during prolonged MV and to develop specific antioxidant countermeasures to protect the diaphragm against the detrimental effects of MV.

CHAPTER 3
METHODS

Animals and Experimental Design

These experiments were approved by the University of Florida Animal Care and Use Committee and followed the guidelines for animal experiments set forth by the National Institutes of Health. Female, Sprague-Dawley rats (4-months old) were randomly assigned to one of five groups, n = 8 per group: 1) acute control, 2) 18 hour spontaneously breathing control (SB), 3) 18 hour SB control with NADPH oxidase inhibition (SBA), 4) 18 hour mechanically ventilated (MV), 5) 18 hour MV with NADPH oxidase inhibition (MVA).

Experimental Protocol

Animals were anesthetized with sodium pentobarbital (60 mg/kg body weight, intraperitoneal (IP)). After reaching a surgical plane of anesthesia, the acute control animals were sacrificed immediately while the SB and MV animals were tracheostomized utilizing aseptic techniques. The SB animals breathed spontaneously for the 18-hour duration while the MV animals were mechanically ventilated with a volume-driven ventilator (Inspira, Harvard Apparatus, Cambridge, MA) for the same duration. The tidal volume was set at approximately 0.55 ml/100 grams body weight with a respiratory rate of 80 breaths per minute and a positive end-expiratory pressure (PEEP) of one centimeter water. In the SBA and MVA animals, the NADPH oxidase inhibitor, apocynin, was dissolved in saline and administered via an IP injection prior to the

experimental protocol (4 mg/kg body weight). This dosage has been previously utilized *in vivo* to inhibit NADPH oxidase activity in skeletal muscle (24).

The carotid artery was cannulated to permit measurement of arterial blood pressure and the collection of blood. During the first hour of the experimental protocol, after approximately 9 hours and during the final hour of the experiment, blood samples were analyzed for the partial pressures of O_2 and CO_2, arterial pH, sodium (Na^+), potassium (K^+), calcium (Ca^{++}), glucose and lactate using an electronic blood gas analyzer (GEM Premier 3000; Instrumentation Laboratory, Lexington, MA). Arterial PO_2 was maintained throughout the experiment by gradually increasing the F_IO_2 using a hyperoxic gas (range 22-25% oxygen) in both SB and MV animals. Moreover, the jugular vein was cannulated for the infusion of saline and sodium pentobarbital (~10 mg/kg body weight/hour). Apocynin was administered via an IP injection (4 mg/kg body weight).

Furthermore, animals received an intramuscular injection of glycopyrrolate (0.04 mg/kg body weight) every two hours to reduce airway secretions. Body temperature was maintained at approximately 37^oC and heart rate was monitored via a lead II electrocardiograph. Continuous care during the experimental protocol included lubricating the eyes, expressing the bladder, removing airway mucus and rotating the animal and limbs of the animal. Enteral nutrition was provided via the AIN-76 rodent diet with a nutrient composition of 15% proteins, 35% lipids, 50% carbohydrates, and vitamins and minerals (Research Diets Inc., New Brunswick, NJ). Our planned feeding schedule was designed to provide an isocaloric diet with the nutrients administered every

two hours with a gastric tube; the total administration of 69 ml is equivalent to 69 kcal/day.

Following the experimental protocol, the diaphragm was quickly removed and placed in a dissecting chamber containing Krebs-Hensleit saline aerated with 95/5% O_2/CO_2. One segment of the costal diaphragm was used to assess the *in vitro* contractile function of the diaphragm while the remaining costal diaphragm was dissected into multiple segments (~ 50 mg) and quickly frozen in liquid nitrogen and stored at –80^{o}C for subsequent assay. Finally, due to the negative effect of sepsis on the diaphragm, blood samples from each animal were cultured to determine if gram positive and gram negative bacteria were present in the blood.

Diaphragmatic Measurements

Contractile Measurements

The force-frequency response of a strip of costal diaphragm was performed and normalized to the cross-sectional area (CSA) as described previously (4). Briefly, a strip of diaphragm muscle was obtained from the midcostal region including the tendinous attachments at the central tendon and rib cage. The strip was vertically suspended in a jacked tissue bath between two plexiglas clamps with one end connected to an isometric force transducer (model FT-03, Grass Instruments, Quincy, MA). The tissue bath was filled with Krebs-Hensleit saline (pH = 7.4) aerated with 95/5% O_2/CO_2.

Following a 15-minute equilibration period at 25^{o}C, the muscle strip was stimulated with platinum electrodes surrounding the muscle strip. A supramaximal stimulation voltage (~150%) was utilized to determine optimal contractile length (Lo) of the muscle strip by systematically adjusting the length of the muscle while stimulating it with single twitches. All contractile measurements were made at Lo.

To determine the force-frequency response, each muscle strip was stimulated with 120 V pulses at 15-160 Hz with a train duration of 500 ms. Contractions were separated by a two minute recovery period. Following this protocol, the muscle strip length was measured at Lo using a caliper. The strip was then trimmed from the supporting rib and all connective tissue and fat was removed. The remaining strip of muscle was weighed and the CSA was determined by using the following formula: total muscle CSA (mm^2) = [muscle mass/(fiber length x 1. 056)]. The density of muscle in g/cm^3 is 1.056 (59).

Biochemical Measurements

NADPH oxidase activity

The activity of NADPH oxidase was measured using a lucigenin technique as described by Cui and Douglas (60). Briefly, muscle was homogenized in a 20 mM potassium phosphate buffer (KPO_4) (pH = 7.2) containing 1 mM EGTA. One hundred microliters of supernatant was added to 900 ul of buffer (50 mM KPO_4, 1 mM EGTA, 150 mM sucrose, 230 uM lucigenin, and 500 uM NADH). The change in luminescence was measured over a 10-minute period and normalized to protein. Protein concentration of the homogenate was determined using the Bradford technique (61).

Myeloperoxidase (MPO) activity

MPO has been shown to be highly correlated with the number of neutrophils present in the tissue and therefore MPO activity was determined as an indication of the level of neutrophil infiltration into the diaphragm during our experiments (62). MPO activity was measured using the method of Seekamp et al. (62). Briefly, muscle was homogenized in a 50 mM KPO_4 buffer containing 0.5% HTAB and 5 mM EDTA (pH = 6.0). Homogenate was sonicated for 10 seconds and centrifuged cold at 3000 x g for 30 minutes. Ten microliters of homogenate was added to 290 ul of the reaction solution (50

mM KPO_4 buffer, 3% H_2O_2, 1% o-dianisidine, pH = 6.0). The change in absorbance was measured for three minutes and the value was expressed as activity units per gram of muscle (wet weight).

Glutathione

As a marker of oxidative stress, reduced glutathione levels were measured in a segment of costal diaphragm using a commercially available kit (Cayman Chemical, Ann Arbor, MI). The principal of this assay is that the sulfhydryl group of glutathione (GSH) reacts with 5,5-dithiobis-2-nitrobenzoic acid (DTNB) to produce a yellow-colored 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide that is produced by this reaction is reduced by glutathione reductase to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to the recycling reaction and, therefore, reflective of the concentration of GSH in the sample. Reduced GSH was calculated by subtracting the amount of oxidized glutathione (GSSG) from the amount of total glutathione. The reduced form of GSH is the most prevalent in biological systems.

Protein carbonyls

The carbonyl assay is a general assay of oxidative damage to proteins. The principle of the assay is that several reactive oxygen species attack amino acid residues in proteins to produce products with carbonyl groups which can be measured after reaction with 2,4-dinitrophenylhydrazine. To measure protein carbonyl levels in our muscle samples, a segment of costal diaphragm was homogenized in phosphate buffered saline (pH = 7.4) and centrifuged at 1000 x g for 30 minutes. The supernatant was adjusted to contain two mg protein/ml and reacted with 2,4-dinitrophenylhydrazine overnight. Protein carbonyl levels in each sample were then detected via a commercially available ELISA kit (Zenith Technology Corporation, Dunedin, NZ).

Nitric oxide synthase (NOS)

Protein levels of all three isoforms of NOS (eNOS, nNOS, iNOS) were detected by Western analysis. Crude muscle homogenate (75 ug protein) was loaded onto a 7.5% tris-glycine SDS polyacrylamide gel and separated via electrophoresis (100 V, 1.5 hours). Proteins were then transferred to nitrocellulose (2 hours at 275 mA) and the membrane was blocked in 5% non-fat dry milk. The membrane was then exposed to a monoclonal antibody for eNOS, nNOS, and iNOS at a dilution of 1:500 (BD Transduction Laboratories, Lexington, KY) followed by exposure to a HRP conjugated anti-mouse secondary antibody. Positive controls were included on each gel including human endothelial cell lysate, rat cerebrum lysate, and mouse macrophage stimulated with IFNγ/LPS for eNOS, nNOS, and iNOS, respectively. Further, membranes were stained with a 0.1% ponceau stain following Western analysis to control for protein loading.

Nitrate and nitrite are the end products of nitric oxide (NO) *in vivo*. Therefore, as an indicator of NOS activity, levels of total nitrate (NO_3-) and nitrite (NO_2-) were measured in the diaphragm with a colorimetric assay kit (Cayman Chemical, Ann Arbor, MI). Briefly, a section of costal diaphragm was homogenized at a 1:10 dilution factor in phosphate buffered saline (pH = 7.4). The homogenate underwent a series of centrifugations including 10,000 x g for 20 minutes, 100,000 x g for 30 minutes, and 12,000 x g with 10K filter tubes (Millipore Corporation, Bedford, MA) for 15 minutes. Next, nitrate was converted to nitrite by utilizing nitrate reductase. Finally, the addition of the Griess reagents converts the nitrite into a deep purple azo compound. Photometric measurement of the absorbance of this compound determines the concentration of nitrite.

3-nitrotyrosine

Protein levels of 3-nitrotyrosine in the insoluble, cytosolic, mitochondrial and membrane fractions were measured via Western analysis using the protocol described by Barreiro et al. (58). Briefly, 100 mg of diaphram muscle was homogenized in a buffer containing 10 mM tris-maleate, 3 mM EGTA, 275 mM sucrose, 0.1 mM DTT, and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) (buffer A). One aliquot of homogenate was used for a dot blot while the remaining homogenate was centrifuged at 1000 x g for 10 minutes. The pellet was then resuspended in buffer A and designated as the insoluble fraction. The supernatant was centrifuged at 12,000 x g for 20 min and the pellet was resuspended in buffer B (10 mM tris-maleate, 0.1 mM EDTA, 135 mM KCl). The supernatant was removed and the pellet was resuspended in buffer A and centrifuged at 12,000 x g for 20 min. The pellet was resuspended in buffer A by sonication and was designated as the mitochondrial fraction. The supernatant from the last two steps was pooled and centrifuged at 100,000 x g for one hour. The supernatant was saved and designated as the cytosolic fraction while the pellet was resuspended in buffer C (10 mM HEPES and 300 mM sucrose) and treated for one hour in 600 mM KCl. The homogenate was then centrifuged at 100,000 x g for one hour. The pellet was resuspended in buffer A by sonication and designated as the membrane fraction. All four fractions were mixed with sample buffer and boiled for five minutes. Protein was loaded on a 4-20% tris-glycine sodium dodecylsulfate (SDS) polyacrylamide gel and separated via electrophoresis (1.5 hours at 100 V). Proteins were then transferred to nitrocellulose (2 hours at 275 mA) and blocked in 1% BSA. The membrane was exposed to a monoclonal antibody for 3-nitrotyrosine (Cayman Chemical, Ann Arbor, MI) followed by exposure to a HRP conjugated anti-mouse secondary antibody. The presence of proteins was

detected using chemiluminescence. Membranes were incubated in SYPRO ruby protein blot stain following Western analysis to control for protein loading. Levels of 3-nitrotyrosine were normalized to the associated protein band.

Statistical Analysis

Comparisons between groups were made by a one-way analysis of variance (ANOVA) and, when appropriate, a Tukey HSD test was performed post hoc. Significance was established at $p < 0.05$.

CHAPTER 4
RESULTS

Systemic and Biologic Response to MV

Heart rate (HR) and systolic blood pressure (BP) were maintained within a physiologic range during the MV protocol (HR = 300 - 420 beats per minute; BP = 70 - 130 mm Hg). Arterial pH, the partial pressures of O_2 and CO_2, and lactate levels were maintained within a normal range during the MV protocol (table 1). Only the SB animals experienced mild hypercapnia and acidosis due to the anesthetic (table 1). Nonetheless, this arterial pH disturbance did not compromise any of the diaphragmatic contractile properties (see results). Sodium (Na^+), potassium (K^+), calcium (Ca^{++}), and glucose levels were also maintained during the SB and MV protocol (table 1). Further, there were no significant differences in body weight between the groups prior to the experimental protocol and the 18-hour experimental protocol did not alter body weight in any of the groups. This indicates that our hydration and nutrition regimen was adequate. Also, note that none of the SB or MV animals tested positive for gram-positive or gram-negative bacteria and there were no visual abnormalities of the lungs or peritoneal cavity. These results indicate that our aseptic surgical technique was successful in preventing infection.

Contractile Dysfunction

To determine if apocynin prevented MV-induced diaphragmatic contractile deficits, we measured diaphragmatic contractile function in diaphragm strips *in vitro*. Figure 1 illustrates the diaphragmatic force-frequency relationship for all five

experimental groups. Eighteen hours of MV resulted in a significant reduction ($p < 0.05$) in the specific force of the diaphragm compared to all other groups at all stimulation frequencies except 15 hertz. However, treatment with apocynin attenuated all of the MV-induced diaphragmatic dysfunction. Also note that diaphragmatic contractile function in both the SB and the SBA animals did not differ from controls.

NADPH Oxidase and MPO Activity

Table 2 contains the values for diaphragmatic NADPH oxidase activity. MV did not result in an increase in NADPH oxidase activity and apocynin did not reduce the activity below CON values. Importantly, in the absence of the substrate for skeletal muscle NADPH oxidase (NADH), there was almost no activity (0.8 RLU/mg protein). Also, the addition of superoxide dismutase (SOD) lowered the activity of control diaphragm homogenate by greater than 60% while the activity of a positive control (rat liver homogenate) was 20 fold greater than that of control diaphragm. Therefore, this data indicates that our assay was highly specific for NADPH oxidase superoxide production and was not detecting superoxide from other sources. Finally, no significant differences were observed between groups for myeloperoxidase (MPO) activity (table 2).

Glutathione

Glutathione is the major non-protein thiol in cells and is considered to be the most important intracellular antioxidant. MV resulted in a significant reduction in the amount of reduced glutathione (GSH) in the diaphragm, indicative of oxidative stress. Note, however, that diaphragmatic levels of GSH did not differ between the CON and MVA groups (figure 2). Therefore, it appears that apocynin attenuated the MV-induced oxidative stress in the diaphragm during MV.

Protein Carbonyls

Protein carbonyl levels were measured as a general index of protein oxidation in the diaphragm. Compared to all other experimental groups (CON, SB, SBA, and MVA), 18 hours of MV resulted in a significant elevation in diaphragmatic protein carbonyl levels (figure 3). Indeed, note that the *in vivo* administration of apocynin prevented the MV-induced increase in protein oxidation in the diaphragm during MV (figure 3).

Nitric Oxide Synthase (NOS)

MV did not result in a change in diaphragmatic protein levels of eNOS or nNOS (table 3). Further, iNOS was not detected in the diaphragm of any of the experimental groups. Representative Western blots are illustrated in figure 4. Finally, MV did not result in an increase in NOS activity as measured by the levels of nitrate and nitrite in the diaphragm (table 4).

3-Nitrotyrosine

3-nitrotyrosine is considered to be the primary end product of nitric oxide (NO) interaction with proteins. No significant differences in diaphragmatic levels of 3-nitrotyrosine existed between experimental groups in crude homogenate or any of the cellular fractions of diaphragmatic muscle (table 5-9). Representative Western blots of these results are illustrated in figure 5.

Table 1. Arteriole blood values during SB and MV protocol.

	pH	pCO_2 (mmHg)	pO_2 (mmHg)	Na^+ (mM)	K^+ (mM)	Ca^{++} (mM)	Glucose (mg/dl)	Lactate (mM)
SB	7.35 ± 0.01	48.7 ± 2.3	62.6 ± 2.9	144.9 ± 0.50	3.47 ± 0.11	1.08 ± 0.02	95.3 ± 4.48	0.51 ± 0.08
MV	7.46 ± 0.01	31.9 ± 1.4	68.5 ± 1.6	143.6 ± 0.53	3.45 ± 0.14	1.05 ± 0.02	96.9 ± 5.37	0.90 ± 0.25
SBA	7.31 ± 0.01	54.2 ± 2.5	71.9 ± 5.1	142.4 ± 0.57	3.55 ± 0.13	1.08 ± 0.03	89.2 ± 5.22	0.47 ± 0.08
MVA	7.41 ± 0.01	35.0 ± 1.7	69.9 ± 2.7	146.5 ± 1.14	3.15 ± 0.09	1.04 ± 0.01	76.3 ± 4.52	0.44 ± 0.06

Values are expressed as mean ± SEM of the pre, mid and post blood gas samples. CON = control; SB = spontaneously breathing; MV = mechanically ventilated; SBA = SB with apocynin; MVA = MV with apocynin.

Table 2. NADPH oxidase and myeloperoxidase (MPO) activity in the diaphragm.

	CON	SB	MV	SBA	MVA
NADPH Oxidase (RLU/mg protein)	392 ± 29	413 ± 23	438 ± 38	354 ± 27	440 ± 42
MPO (U/gww)	1.17 ± 0.069	1.33 ± 0.097	1.22 ± 0.053	1.21 ± 0.046	1.19 ± 0.063

Values are expressed as mean ± SEM. No significant differences were detected between any experimental groups. CON = control; SB = spontaneously breathing; MV = mechanically ventilated; SBA = SB with apocynin; MVA = MV with apocynin; RLU = relative light units; U/gww = units per gram wet weight.

Table 3. Nitric oxide synthase (NOS) protein levels in the diaphragm.

	CON	SB	MV
eNOS	100.0 ± 8.6	105.2 ± 7.8	91.7 ± 9.9
nNOS	100.0 ± 11.3	108.2 ± 12.2	104.1 ± 14.7

Values are expressed as a percent of control (mean ± SEM). No significant differences were detected between any of the experimental groups. CON = control; SB = spontaneously breathing; MV = mechanically ventilated; eNOS = endothelial nitric oxide synthase; nNOS = neuronal NOS.

Table 4. Nitrate and nitrite levels in the diaphragm.

	CON	SB	MV
Nitrate and nitrite (μmol/gww)	105.5 ± 9.6	104.5 ± 5.8	99.4 ± 5.9

Values are expressed as mean ± SEM. No significant differences were detected between any experimental groups. CON = control; SB = spontaneously breathing; MV = mechanically ventilated.

Table 5. Diaphragmatic protein levels of 3-nitrotyrosine in crude homogenate.

CON	SB	MV
100.0 ± 5.9	97.3 ± 5.2	98.6 ± 4.0

Values are expressed as a percent of control (mean ± SEM). No significant differences existed between any of the experimental groups. CON = control; SB = spontaneously breathing; MV = mechanically ventilated.

Table 6. Diaphragmatic levels of 3-nitrotyrosine within insoluble proteins.

~ Molecular Wt	CON	SB	MV
200 kDa	100.0 ± 7.9	94.8 ± 9.0	88.4 ± 5.7
95 kDa	100.0 ± 11.9	108.3 ± 11.9	96.9 ± 10.0
80 kDa	100.0 ± 5.8	96.7 ± 11.2	109.3 ± 10.3
40 kDa	100.0 ± 9.4	93.9 ± 13.0	102.4 ± 8.2
30 kDa	100.0 ± 9.0	84.7 ± 8.4	109.1 ± 9.7

Values are expressed as a percent of control (mean ± SEM). No significant differences existed between any of the experimental groups. CON = control; SB = spontaneously breathing; MV = mechanically ventilated.

Table 7. Diaphragmatic protein levels of cytosolic (soluble) 3-nitrotyrosine.

~ Molecular Wt	CON	SB	MV
40 kDa	100.0 ± 7.4	89.5 ± 9.3	86.8 ± 5.3
27 kDa	100.0 ± 6.8	117.9 ± 5.9	109.3 ± 11.1

Values are expressed as a percent of control (mean ± SEM). No significant differences existed between any of the experimental groups. CON = control; SB = spontaneously breathing; MV = mechanically ventilated.

Table 8. Diaphragmatic mitochondrial protein levels of 3-nitrotyrosine.

~ Molecular Wt	CON	SB	MV
70 kDa	100.0 ± 14.6	104.8 ± 13.5	90.5 ± 6.2
40 kDa	100.0 ± 11.9	100.2 ± 13.8	106.0 ± 16.6
22 kDa	100.0 ± 12.4	107.2 ± 7.2	116.1 ± 14.5

Values are expressed as a percent of control (mean ± SEM). No significant differences existed between any of the experimental groups. CON = control; SB = spontaneously breathing; MV = mechanically ventilated.

Table 9. Diaphragmatic protein levels of 3-nitrotyrosine within membrane proteins.

~ Molecular Wt	CON	SB	MV
70 kDa	100.0 ± 10.8	87.8 ± 11.2	102.7 ± 12.8
40 kDa	100.0 ± 14.1	106.1 ± 17.3	100.0 ± 7.6
22 kDa	100.0 ± 13.6	98.7 ± 12.0	108.8 ± 8.5

Values are expressed as a percent of control (mean ± SEM). No significant differences existed between any of the experimental groups. CON = control; SB = spontaneously breathing; MV = mechanically ventilated.

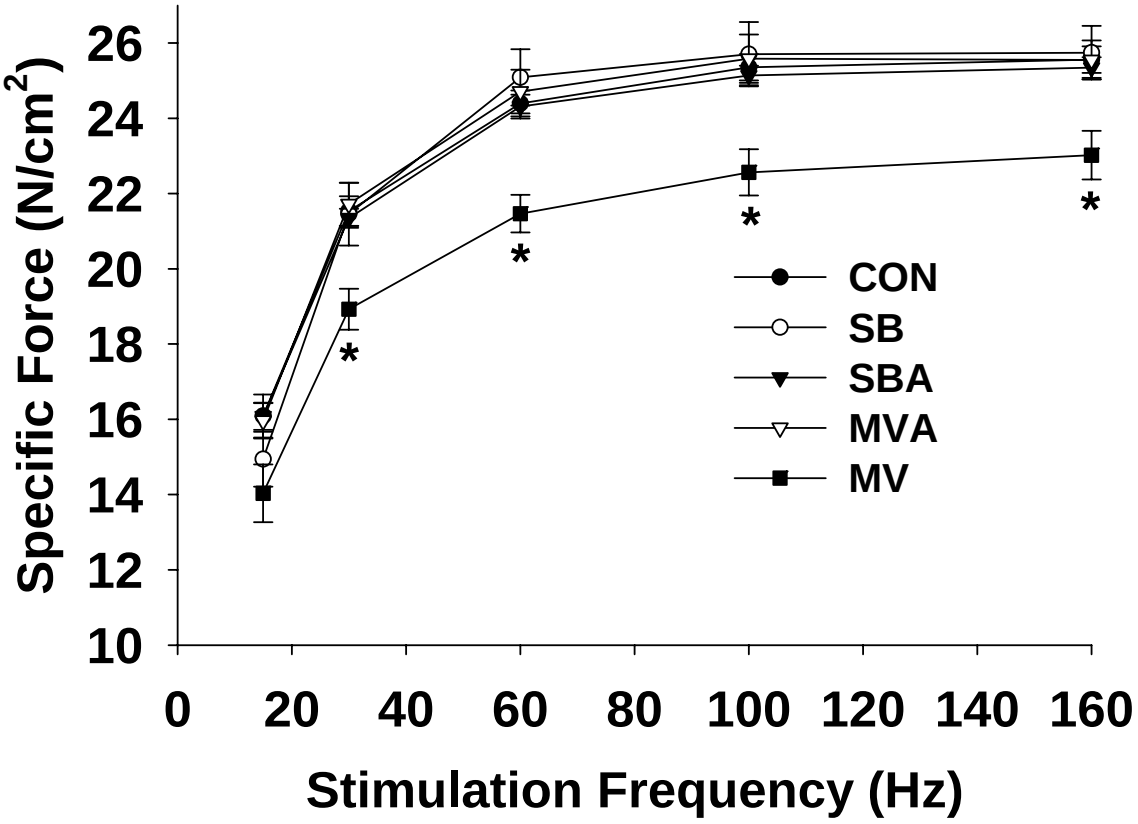

Figure 1. Effect of 18 hours of mechanical ventilation (MV) on the diaphragmatic force-frequency response. Values are expressed as mean ± SEM. * MV significantly different versus all other groups ($p < 0.05$). CON = control; SB = spontaneously breathing; MV = mechanically ventilated; SBA = SB with apocynin; MVA = MV with apocynin.

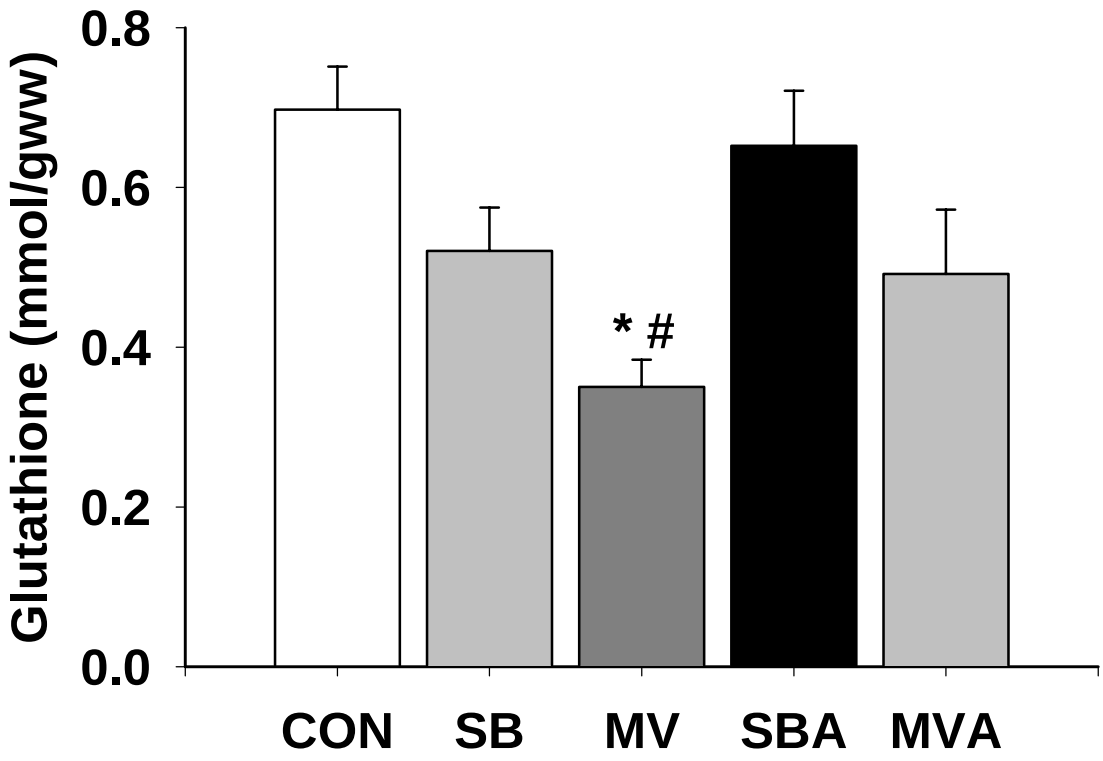

Figure 2. Effect of 18 hours of mechanical ventilation on the diaphragmatic levels of reduced glutathione. Values are expressed as mean ± SEM. * Significantly different versus CON ($p < 0.05$); # Significantly different versus SBA ($p < 0.05$). Note that the MVA group did not differ from control. CON = control; SB = spontaneously breathing; MV = mechanically ventilated; SBA = SB with apocynin; MVA = MV with apocynin.

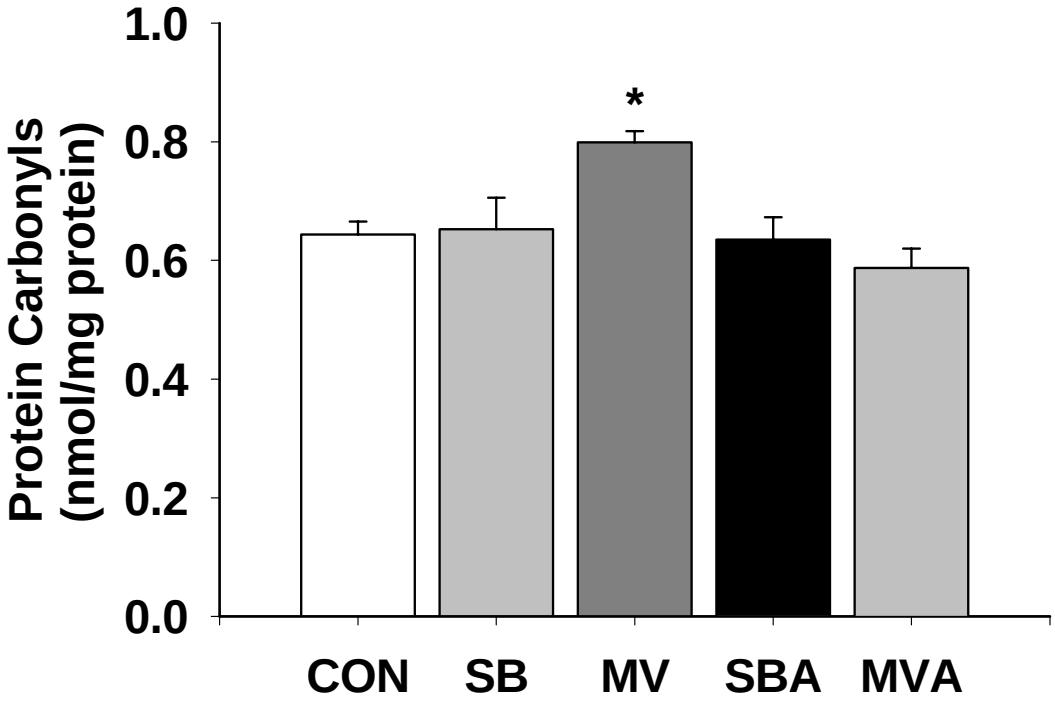

Figure 3. Effect of 18 hours of mechanical ventilation on the diaphragmatic levels of protein carbonyls. Values are expressed as mean ± SEM. * MV significantly different versus all other groups ($p < 0.05$). Note that the MVA group is significantly lower than the MV group. CON = control; SB = spontaneously breathing; MV = mechanically ventilated; SBA = SB with apocynin; MVA = MV with apocynin.

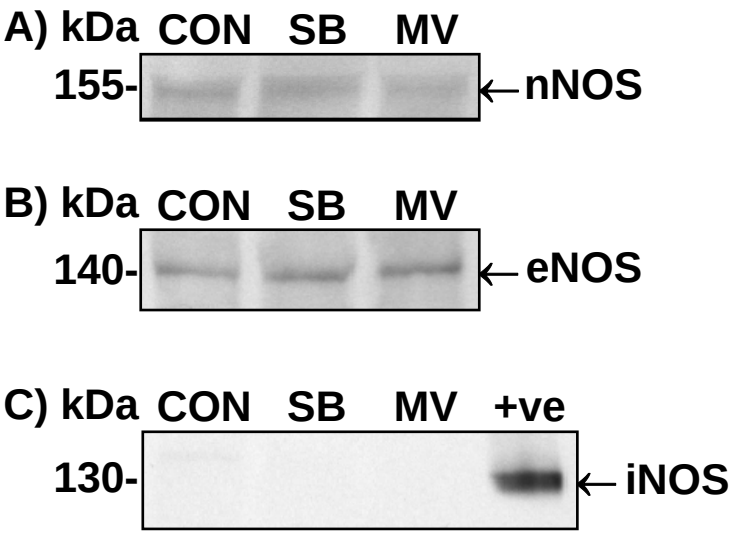

Figure 4. Representative western blots illustrating the diaphragmatic protein levels of NOS. A) nNOS, B) eNOS, C) iNOS. CON = control; SB = spontaneously breathing; MV = mechanically ventilated; +ve = positive control for iNOS (mouse macrophage exposed to IFN-γ and LPS).

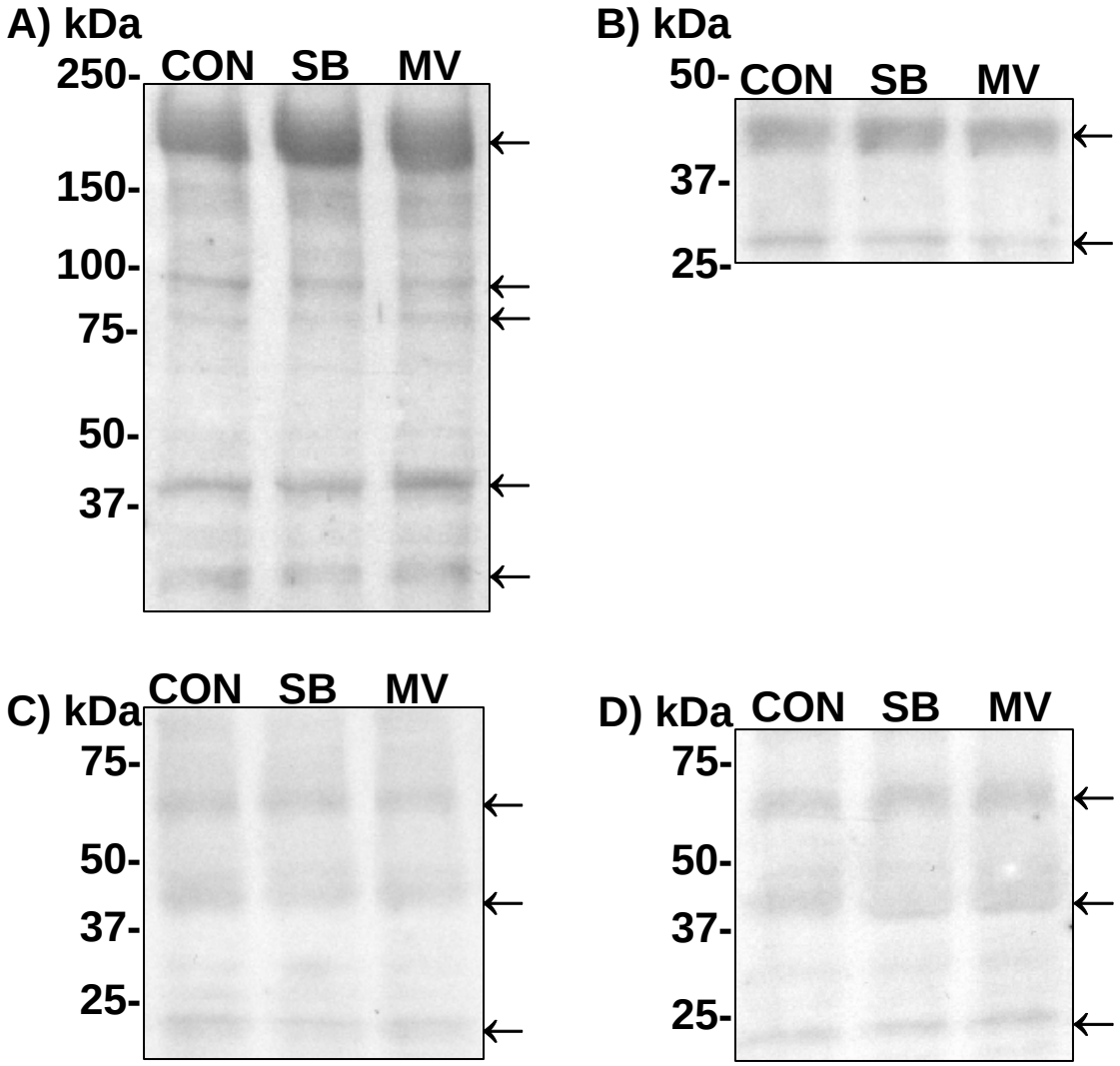

Figure 5. Representative western blots illustrating the diaphragmatic protein level of 3-nitrotyrosine. A) insoluble fraction, B) cytosolic fraction, C) mitochondrial fraction, D) membrane fraction. Arrows indicate positive band for 3-nitrotyrosine. CON = control; SB = spontaneously breathing; MV = mechanically ventilated.

CHAPTER 5
DISCUSSION

Overview of Principal Findings

Several important findings emerged from these experiments. First, these results indicate that MV-induced oxidative injury to the diaphragm is not due to an increase in NADPH oxidase activity. Secondly, these experiments reveal that the *in vivo* administration of apocynin attenuates the contractile dysfunction and oxidative stress induced by MV; however, this amelioration was not due to the inhibition of NADPH oxidase and appears to be due to the antioxidant properties of apocynin. Finally, MV is not associated with an increase in protein levels of any of the three NOS isoforms (eNOS, nNOS, or iNOS), an increase in NOS activity or an increase in the accumulation of 3-nitrotyrosine within the diaphragm. A brief discussion of these results follows.

Role of NADPH Oxidase in MV-induced Oxidative Stress

Our first hypothesis for the current experiment was that NADPH oxidase activity is increased in the diaphragm during MV and contributes to diaphragmatic oxidative injury and contractile dysfunction. This postulate was based on data from our laboratory indicating that prolonged MV promotes an increase in total calcium levels (unpublished observations) and activation of calcium-activated neutral proteases (calpain) in the diaphragm (10). Since NADPH oxidase is a calcium-activated enzyme, we postulated that increases in diaphragmatic free calcium could up-regulate NADPH oxidase activity. Nonetheless, our data do not support the postulate that MV is associated with an increase in NADPH oxidase activity in the diaphragm following MV.

It is important to note that NADPH oxidase exists in two isoforms and that reactive oxygen species (ROS) can be produced by both phagocytic and non-phagocytic isoforms of NADPH oxidase (63). A previous investigation from our laboratory has shown that MV is not associated with an increase in phagocytic cells (35). These previous findings agree with the current investigation in that MV did not result in an increase in myeloperoxidase (MPO) activity in the diaphragm; MPO is considered to be an excellent marker of neutrophil infiltration (62). Therefore, based on these collective findings, we conclude that phagocytic NADPH oxidase activity does not increase and apparently does not contribute to MV-induced diaphragmatic dysfunction.

The non-phagocytic NADPH oxidase found in skeletal muscle is similar to the phagocytic isoform, however some differences do exist. The phagocytic isoform of NADPH oxidase is composed of three cytosolic subunits ($p47^{phox}$, $p67^{phox}$, and $p40^{phox}$) and a membrane-spanning cytochrome b_{558} composed of two subunits ($p22^{phox}$ and $gp91^{phox}$). Upon activation, there is a migration of the cytosolic subunits to the membrane-spanning cytochrome. In comparison, a recent investigation by Javesghani et al. has characterized the non-phagocytic NADPH oxidase enzyme complex in rat skeletal muscle (22). They determined that only four of the five subunits exist in skeletal muscle ($p22^{phox}$, $gp91^{phox}$, $p47^{phox}$, and $p67^{phox}$) and they are all constitutively expressed on the membrane. Therefore, non-phagocytic NADPH oxidase activation does not require the translocation of subunits from the cytosol to the membrane. Interestingly, the mechanism by which apocynin inhibits phagocytic NADPH oxidase activity is most likely due to the inhibition of the enzyme assembly by blocking sulfhydryl groups (23). This could

explain, at least in part, why there was no inhibitory effect of apocynin on skeletal muscle NADPH oxidase activity in the current experiments (table 2).

Our decision to use apocynin as an NADPH oxidase inhibitor was based on previous investigations showing its inhibitory effect on NADPH oxidase in skeletal muscle (22, 24). However, during the course of the current experiment, a new report appeared in the literature suggesting that apocynin has limited efficacy as a non-phagocytic NADPH oxidase inhibitor (64). This report revealed that the *in vitro* addition of apocynin inhibited ROS formation in phagocytic cells (i.e., macrophages) but was not effective in the inhibition of NADPH oxidase activity in non-phagocytic cells (i.e., vascular fibroblasts).

If apocynin does not inhibit NADPH oxidase activity in skeletal muscle, why does the administration of apocynin prevent MV-induced diaphragmatic contractile dysfunction? A definitive answer to this question is unavailable but it is possible that apocynin acts as an intracellular antioxidant to retard MV-induced oxidative stress and protect against oxidant-mediated diaphragmatic contractile dysfunction. Indeed, the molecular structure of apocynin contains a phenol group with oxidant scavenging capacity. The likelihood that apocynin is a physiological antioxidant could explain our finding that treatment with apocynin prevented both MV-induced protein oxidation and diaphragmatic contractile dysfunction. The possibility that apocynin is a physiologically useful antioxidant warrants further investigation to determine if this compound is a clinically useful countermeasure to prevent MV-induced diaphragmatic oxidative stress and contractile dysfunction.

Role of Nitric Oxide Synthase (NOS) in MV-induced Oxidative Stress

The second hypothesis tested in this investigation was that NOS levels are elevated during MV, increasing the nitration of diaphragmatic proteins. This postulate was formulated from our preliminary data indicating that MV results in an increase in total calcium in the diaphragm and knowledge that both of the constitutive isoforms of NOS (eNOS and nNOS) are calcium-activated enzymes that produce NO. Therefore, an MV-induced increase in calcium could activate one or both of these NOS isoforms leading to the formation of NO and the nitration (e.g., 3-nitrotyrosine) of diaphragmatic proteins. Nonetheless, our results revealed that 18 hours of MV was not associated with an increase in diaphragmatic levels NOS or 3-nitrotyrosine.

Our finding that MV-induced inactivity in the diaphragm does not alter the levels of any NOS isoform differs from a previous report by Nguyen and Tidball indicating that nNOS levels decrease in mice locomotor skeletal muscle following 10 days of hindlimb unloading (41). Changes in muscle levels of nNOS could be physiologically significant because an increase in NO in inactive skeletal muscle can be beneficial by inhibiting calpain and, therefore, reducing the degradation of cytoskeletal proteins such as talin and vinculin (65). Note, however, although no changes in nNOS levels occurred during 18 hours of MV in the current study, longer periods of MV may decrease levels of nNOS leaving the diaphragm more susceptible to degradation by calpain.

The finding that MV is not associated with an increase in the levels of 3-nitrotyrosine in the diaphragm suggests that NO production was not accelerated in the diaphragm during MV. Our analysis of 3-nitrotyrosine levels in the diaphragm was comprehensive and evaluated the nitration of proteins in a wide variety of protein pools in the cell including the insoluble, cytosolic, mitochondrial, and membrane protein

fractions of diaphragm fibers following MV. This type of comprehensive analysis is important to detect small treatment-induced changes in 3-nitrotyrosine because a previous investigation by Barreiro et al. has revealed that the nitration of muscle proteins can be limited to one or two protein compartments of the muscle fiber (58). For example, these investigators determined that sepsis-induced nitration of proteins was limited to the membrane and mitochondrial fractions of diaphragm (58). Therefore, the failure to separate muscle proteins into sub-fractions could mask increases in nitration within small pools of protein within the fiber. However, based upon our comprehensive analysis of 3-nitroyrosine in the diaphragm, the current results indicate that 18 hours of MV is not associated with an increase in the nitration of diaphragmatic proteins.

Critique of Experimental Model

Obtaining a diaphragm muscle biopsy is invasive; therefore, a non-human model must be utilized to study the effects of MV on the diaphragm. The current investigation utilized the rat as the experimental model for two reasons: 1) the animal size permits the necessary surgical techniques to be conducted and also allows for the removal of several arterial blood samples which is necessary for the maintenance of blood gas homeostasis, and more importantly, 2) human and rat diaphragms are similar anatomically and functionally as well as having similar fiber type composition (7, 8).

Controlled MV was utilized in the current study versus pressure-assist MV. Complete inactivity of the diaphragm results from controlled MV and is utilized clinically in cases of drug overdose, spinal cord injury, surgery and is also common in pediatric patients (3).

Sodium pentobarbital was administered as the anesthetic because the current and previous investigations clearly indicate that sodium pentobarbital does not compromise

the function of the diaphragm (4, 10, 21). Apocynin was utilized in the current investigation because previous studies have reported that it is an effective inhibitor of NADPH oxidase in skeletal muscle (22, 24). Another common inhibitor of NADPH oxidase, diphenylene iodonium (DPI), was not utilized in these experiments because this compound has been shown to be non-selective for NADPH oxidase. Indeed, DPI has been shown to inhibit multiple flavoproteins (66) including NADPH oxidase (67, 68), NOS (69), xanthine oxidase (70), mitochondrial NADH-ubiquinone oxidoreductase (71, 72), and cytochrome p450 (73).

Conclusions

These are the first experiments to investigate the sources of oxidant production in the diaphragm during prolonged MV. Our studies reveal that MV-induced diaphragmatic dysfunction and oxidative stress is not due to an increase in NADPH oxidase activity. Moreover, the *in vivo* administration of apocynin attenuates the diaphragmatic contractile dysfunction and oxidative stress induced by MV via its antioxidant properties. Furthermore, MV does not promote an increase in any of the NOS isoforms, NOS activity or cause the nitration of proteins in the diaphragm. Therefore, it can be concluded that neither NADPH oxidase nor NOS contributes to diaphragmatic contractile dysfunction and oxidative stress that occurs during MV.

Future Directions

The current investigation provides evidence that NADPH oxidase and NOS are not involved in MV-induced diaphragmatic oxidant stress and contractile dysfunction. However, several other sources of oxidants exist in skeletal muscle fibers. For example, the mitochondria can produce superoxide at complex I and III along the electron transport chain (44). Although the investigation of mitochondrial oxidants in the diaphragm during

prolonged mechanical ventilation is complicated, use of mitochondrial targeted antioxidants could be a useful probe to study this phenomenon.

Moreover, increases in calcium activated neutral proteases (calpain) can up-regulate xanthine oxidase activity resulting in the formation of excess superoxide production (44). Our laboratory has reported an increase in calpain activity during MV (10). Therefore, the increase in calpain activity seen during MV may lead to the production of superoxide via xanthine oxidase. Future experiments using specific pharmacological probes to inhibit xanthine oxidase activity could prove useful in investigating the role of this oxidant producing pathway.

Finally, skeletal muscle atrophy is associated with an increase in free, “reactive” iron (48). Free iron can react with hydrogen peroxide and superoxide to produce the highly reactive hydroxyl radical (44). Therefore, it is possible that MV-induced increases in free iron could be a source of reactive oxygen species in the diaphragm (10). A variety of membrane permeable iron chelating compounds exist and be useful in investigating the role that reactive iron plays in MV-induced oxidant production in the diaphragm. Determining which of the potential sources of oxidant production is involved in MV-induced oxidative stress is important and could provide a therapeutic intervention to retard MV-induced diaphragmatic contractile dysfunction.

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BIOGRAPHICAL SKETCH

Darin Lee Van Gammeren was born in Luverne, Minnesota, and raised on a farm in rural Inwood, Iowa. He graduated from the University of Sioux Falls, Sioux Falls, South Dakota, with a bachelor's degree in exercise science. In 1999 he moved to Kearney, Nebraska, where he attended the University of Nebraska at Kearney and received a master's degree in exercise science. Darin began work on a Doctor of Philosophy degree in exercise physiology in 2001 at the University of Florida. Darin will begin post-doctoral training at Boston University in the area of skeletal muscle disuse atrophy under the direction of Dr. Susan Kandarian in July 2005.