OVEREXPRESSION OF HO-1 ATTENUATES MV-INDUCED ATROPHY OF THE DIAPHRAGM

By

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To the numerous teachers and professors who contributed to my education and to my wife, friends and family for their continuous support

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Mechanical ventilation (MV) is the primary means of support used in patients with respiratory muscle failure. The removal of patients from MV is termed 'weaning' and problems in weaning occur frequently. Importantly, weaning difficulties are attributed to diaphragmatic weakness characterized by atrophy and contractile dysfunction. Oxidative stress contributes to MV-induced diaphragmatic weakness. However, the sequence of events leading to oxidative stress has not been fully elucidated. A key stress sensitive enzyme, heme oxygenase-1 (HO-1), is rapidly induced in the diaphragm during MV. Paradoxically, HO-1 may function either as a pro- or antioxidant and the role that HO-1 plays in MV-induced oxidative stress in the diaphragm is unknown. To address this question we mechanically ventilated rats for 12 or 18 hours (MV) with subsets of animals that combined MV along with a HO-1 inducing agent, hemin (MVH); or MV with a HO-1 activity inhibitor, CrMPIX (MVI). Indices of oxidative stress, proteolytic activation, and atrophy were measured in the diaphragm following the experimental protocol. Our study reveals that hemin-induced elevation of HO-1 during MV provides protection against oxidative injury, proteolytic activation, and diaphragmatic atrophy. Specifically, hemin administration preserves glutathione levels and prevents lipid peroxidation in the diaphragm during MV. Further, overexpression of HO-1 decreased the MV-induced activation of calpain

and caspase-3 in the diaphragm. Finally, the hemin-induced resistance to oxidative stress and protease activation in the diaphragm, led to an attenuation of diaphragmatic atrophy following 18 hours of MV. We conclude that HO-1 does not function as a pro-oxidant in the diaphragm but instead provides antioxidant and cytoprotective properties during MV.

CHAPTER 1
INTRODUCTION

A variety of conditions including spinal cord injury, drug overdose, surgery and periods of unconsciousness render the pulmonary system unable to sustain blood gas homeostasis. Positive pressure mechanical ventilation (MV) provides an unmatched superiority in sustaining adequate alveolar ventilation in these patient populations (39). Unfortunately, removal from the ventilator (weaning) is inherently difficult, accounting for more than 40% of the total time spent on the ventilator (27). In fact, approximately 25% out of all those requiring MV experience weaning difficulties translating to billions of dollars per year spent in weaning procedures (61).

In order to elucidate the potential mechanisms of MV-induced weaning difficulties, our laboratory and others have highlighted the link between oxidative stress and diaphragmatic complications (15, 28, 42, 100, 119, 129). The generation of reactive oxygen species (ROS) in the diaphragm during MV has several targets within diaphragm fibers (proteins, lipids, DNA) and contributes to their dysfunction(129). This occurs within 6 hours following the initiation of MV and oxidation of the key contractile proteins actin and myosin has been reported (129). Additionally, oxidative stress promotes the activation of skeletal muscle proteases(85, 97). Pilot data from our laboratory indicates that the calpain and caspase-3 proteases are critical oxidative stress sensitive components in the development of atrophy. Consequently, these events may contribute to the MV-induced decrease in the diaphragmatic specific force production and cross-sectional area of diaphragm fibers.

In this regard, we have focused on heme oxygenase-1 (HO-1) as a potential regulator of redox control in the diaphragm during MV. HO-1 is an intracellular enzyme localized primarily to the microsomal fraction of the cell and is considered to illicit antioxidant properties (47). The enzyme catalyzes the rate-limiting step in the degradation of heme resulting in the generation of

carbon monoxide, biliverdin and free iron (Fe^{2+}). Importantly, biliverdin is further reduced to bilirubin via biliverdin reductase exhibiting potent antioxidant effects. While the induction of the iron sequestering protein ferritin is closely associated with HO-1 accumulation, the possibility of radical formation exists due to the release of iron (7, 9, 58, 77, 92, 110). Consequently, it is yet to be established whether HO-1 functions as a pro- or antioxidant in the diaphragm during MV. Therefore, these experiments investigated the role HO-1 plays in maintaining redox balance and minimizing subsequent protease activation in the diaphragm during MV. Our experiments were designed to achieve the following specific aims.

Specific Aim 1: To determine if pharmacological induction of HO-1 expression in the diaphragm protects against MV-induced oxidative damage.

Rationale: Our work indicates that oxidative stress plays a prominent role in the development of diaphragmatic injury during MV. It is also known that oxidative stress increases HO-1 in a variety of cell types. In addition, prior induction of HO-1 affords protection in a variety of cell types against ensuing periods of oxidative stress.

Hypothesis: Increased HO-1 expression in the diaphragm will attenuate oxidative stress during MV.

Specific Aim 2: To determine if elevated levels of HO-1 will protect against MV-induced activation of calpain and caspase-3 in the diaphragm during prolonged MV.

Rationale: Preliminary data from our laboratory indicate the proteases, calpain and caspase-3 as critical initiators of the proteolytic cascade in the diaphragm during MV. Further, it appears likely that MV-induced oxidative stress is a prerequisite for the activation of calpain and caspase-3. However, it is unknown whether HO-1 can mediate protection against protease activation in skeletal muscle by maintaining redox homeostasis.

Hypothesis: An increase of HO-1 in the diaphragm will attenuate MV-induced protease activation (i.e., calpain and caspase-3) and protect against MV-induced diaphragmatic atrophy.

Specific Aim 3: To investigate the dose-response relationship between HO-1 levels and protection against reactive oxygen species-induced oxidative injury in skeletal muscle myotubes.

Rationale: In various cell types, high levels of HO-1 confer protection against oxidative stress. However, it is unknown whether prior induction of HO-1 improves or exacerbates the effects of oxidative stress in skeletal muscle myotubes. By utilizing a murine C2C12 myogenic model we will determine the importance of HO-1 for cellular protection against oxidative stress.

Hypothesis: HO-1 acts as an antioxidant in skeletal muscle and protects against oxidative injury.

CHAPTER 2
LITERATURE REVIEW

The diaphragm is the primary muscle of respiration in mammals and is necessary for normal breathing. In this regard, it is clear any perturbation to the diaphragm will have a major impact on the overall health of the individual. Because MV is associated with respiratory muscle weakness and subsequent weaning difficulties, elucidating the mechanisms of diaphragmatic injury is critical. Defining the possible mechanisms of protection against MV-induced diaphragmatic injury plays a pivotal role in defining optimal therapeutic strategies in the intensive care unit.

A multitude of factors can contribute to MV-induced diaphragmatic injury. In order to focus the scope of this section, the role of oxidative stress and its contribution to diaphragmatic weakness will be discussed. Therefore, the objectives of this review are to characterize the diaphragmatic injury associated with MV and then discuss the potential sources of oxidative injury.

The second objective of this review will discuss the role of the enzyme responsible for the degradation of heme: heme oxygenase-1 (HO-1). Though HO-1 has been primarily purported in the literature to serve as an antioxidant it has the potential to function as a pro-oxidant. Therefore, this section will elaborate on the evidence supporting both of these notions to discern whether HO-1 serves as a pro- or antioxidant.

MV Induced Myofiber Injury

Characteristics of Myofiber Injury

Respiratory muscle failure is characterized by periods of respiration where an individual is unable to maintain sufficient alveolar ventilation. MV is used in order to achieve adequate ventilation, however difficulties are often succumbed when cessation of ventilator use (weaning)

is deemed appropriate(61). An overwhelming breadth of evidence indicates that weaning difficulties are attributed to MV induced changes within the diaphragm (2, 15, 21, 22, 28, 31, 32, 42, 60, 81, 86-88, 94, 95, 98-100, 119, 127, 129, 131). Currently, there is extensive data from four animal models (rats, rabbits, pigs and baboons) along with limited data from human research which have begun to define the effects of MV on the diaphragm.

Importantly, all models of MV have illustrated a wide array of deleterious effects on the diaphragm(2, 50, 60, 62, 86, 88, 94, 124)**.** Specifically, rodent studies have observed decreased diaphragmatic performance during periods of MV lasting from 12-48 hours (15, 60, 86, 93-95, 98, 119). Until recently, it was unclear whether the diaphragmatic dysfunction resulting from MV was similar in human patients. This is no longer a mystery, due to the novel approach undertaken by Levine and colleagues. Their investigations examined the response of the human diaphragm between 18-72 hours of MV. They observed an approximate 40% decrease in cross-sectional area across both fiber-types(62). Additional evidence from human investigations has shown a 50% drop in twitch transdiaphragmatic pressure in those with diseases as well as small decreases in cross-sectional area in a neonatal population following MV(50, 124). Clearly, limited human evidence justifies the importance of using animal studies in defining the mechanisms causing dysfunction and atrophy of the diaphragm resulting in weaning failure.

Mechanisms of Diaphragmatic Dysfunction

Atrophy

Atrophy of the diaphragm has been reported in virtually all animal experiments following MV (2, 14, 31, 60, 71, 72, 127). Although atrophy occurs in a variety of disuse models, MV is unique in that a swift induction of atrophy occurs (i.e. 12 hours) (60, 71, 100, 127). A decrease in protein synthesis (56), an increase in protein degradation (16), or combination of the two can result in disuse mediated atrophy. Our laboratory has observed a decrease in protein synthesis in

as few as 6 hours following MV (99). Further, decreases in protein synthesis-related signaling and depression of type I and IIx myosin heavy chain are evident during 12-24 hours of MV (70, 99). Nonetheless, our past findings suggest the rapid development of diaphragmatic atrophy following MV is primarily due to an increase in proteolytic-related mechanisms (22, 71, 100).

Proteolysis

There are several systems that contribute to skeletal muscle degradation during periods of oxidative stress, including the lysosomal, calpain, caspase-3, and proteasome pathways(13, 85, 121). Importantly, the contributions of the lysosomal and proteasome pathways play a minor role in the initial cascade of skeletal muscle breakdown during disuse (reviewed in(85). However, elevated and sustained increases in intracellular calcium can activate both calpain and caspase-3. Both of these proteases have been shown to play a major role in skeletal muscle regulation by cleaving myofilament and cytoskeletal-related proteins (20). Importantly, one of the hallmarks of oxidative stress is disruption of intracellular calcium homeostasis (reviewed in (40). Therefore, cellular redox disturbances could elevate calpain and caspase-3 activities and play a significant role in the progression of proteolysis and dysfunction of diaphragm myofibers during MV (45). By controlling the redox state and limiting the proteolytic cascade of events in the cell it may be possible to attenuate MV-induced diaphragmatic injury.

Structural Injury

Alterations in myofiber structure such as membrane and mitochondrial disruption, appearance of lipid vacuoles in the sarcoplasm, and cytostructural protein degradation have been reported in skeletal muscle disuse models (14, 94). Furthermore, similar finding have been noted in diaphragms from mechanically ventilated animals (120) but it is important to note that hindlimb muscles (i.e. inactive) from mechanically ventilated animals do not exhibit these markers (71, 94). The occurrence of myofiber disruption is noteworthy due to the close

association of myofiber injury and contractile dysfunction (94). Although the exact mechanisms are still unclear, several possibilities do exist. The activation of calpains and/or caspase-3 during MV may be responsible for initiating the cascade of events associated with myofilament degradation (85). Recently several investigation have shown that inhibition of calpain or caspase-3 during MV can decrease proteolysis, atrophy and contractile dysfunction (69, 71). Another possible mechanism for myofiber disruption would include reloading of the muscle following inactivity. Reloading of hindlimb muscles following extended periods of disuse is associated with increased vulnerability to muscle fiber injury (78). Therefore, we carefully control the level of anesthesia during MV to prevent spontaneous breathing by animals which would elicit a "reloading" type effect on the diaphragm. Nonetheless, our laboratory has addressed this issue where animals were ventilated for 24 hours and then allowed to spontaneously breathe for 2 hours. This protocol did not induce further contractile dysfunction, membrane damage, or mediation of inflammatory related species (119).

Oxidative Stress

The role of oxidative stress during skeletal muscle atrophy was demonstrated in the early 1990's through pioneering studies by Kondo(51-55). Since that time, the relationship between oxidative stress and myofiber injury has been well documented (reviewed in(59, 84, 85, 89). Oxidative stress can alter the structure, function and/or regulation of lipids, proteins, and nucleic acids. Due to these features, it is necessary to reveal the sources of oxidants contributing to redox imbalances in the cell. Numerous pathways exist (Fig. 2-1) and may independently or cooperatively contribute to myocyte damage including: 1) xanthine oxidase; 2) nitric oxide synthase (NOS); 3) NADPH oxidase; 4) mitochondrial; and 5) HO-1.

Xanthine Oxidase. Xanthine oxidase (XO) is produced via sulfhydryl oxidation or proteolysis of xanthine dehydrogenase by calcium activated proteases (calpain)(35, 38). In the

presence of oxygen and purine substrates (hypoxanthine, xanthine), XO catalyzes the formation of superoxide radicals and uric acid. Recent evidence indicates that XO is found in skeletal muscle fibers in humans and other animals and contributes to XO mediated oxidant production in the diaphragm during contraction(38, 104). Although, during resistive breathing where oxidant production is evident, it appears the XO pathway plays a minor role(106, 107). Therefore, future investigations concerning XO are warranted in order to determine its contribution to oxidant production in the diaphragm during MV.

NAD(P)H Oxidase. NAD(P)H oxidases are membrane-associated enzymes that catalyze the one-electron reduction of molecular oxygen using either NADH or NADPH as electron donors. Importantly, there is a non-phagocytic and non-mitochondrial NAD(P)H oxidase is found in both human and rodent skeletal muscle(43). This is pertinent to our model where the contribution of sepsis related oxidant production has been minimalized. Numerous factors can increase NAD(P)H oxidase activity in cells including the calcium-sensitive PKC-ERK1/2 pathway and it is possible this pathway plays a role in NAD(P)H oxidase activity in the diaphragm during prolonged MV (37). Our laboratory has found that inhibition of NAD(P)H oxidase (apocynin) lessens MV-induced oxidative stress and contractile dysfunction in the diaphragm (VanGammeren et al).

Nitric Oxide Synthase. Three isoforms of NOS exist(44, 102): 1) inducible NOS (iNOS) is calcium independent; 2) endothelial NOS (eNOS) is calcium activated; and 3) neuronal NOS (nNOS) is also calcium activated. Both nNOS and eNOS are expressed in the diaphragm (102, 118) and iNOS is present during periods of endotoxin stress(11). Endogenous nitric oxide production via NOS can result in the formation of reactive nitrogen species, including peroxynitrite ($ONOO^-$). Reactive nitrogen species are associated with cellular injury including

mitochondrial dysfunction, lipid peroxidation, and nitrosylation of proteins (11, 79, 102, 108). Although, recent work from our lab suggests the contribution of nitric oxide to diaphragmatic dysfunction and injury is minor if even detectable during MV (118).

Mitochondrial oxidants. The mitochondria have the capability to be a potent source of ROS production in the cell although (17, 84) (1). While the electron transport chain primarily functions to produce ATP, however, an electron 'leak' may occur where side reactions produce superoxide, which is further dismutated to hydrogen peroxide. In addition, due to the abundant production of oxidants in the mitochondrial matrix and cytosol, the outer membrane also contributes to hydrogen peroxide generation. Clearly, the mitochondria have the potential to shift cellular redox balance and during periods of sepsis and their role in cellular injury has been demonstrated in the diaphragm (109). However, the role of mitochondrial generated oxidants in the diaphragm is unclear in sepsis-free conditions where negligible effects were observed after 5 days of MV(30).

Heme Oxygenase-1. The observation of the endogenous colored pigments biliverdin and bilirubin formed via the degradation of heme have been known for over 50 years but the enzymatic reactions responsible were not discovered until many years later(113-115). Tenhunen et al. was first to illustrate the effects of the enzyme, heme oxygenase, which is responsible for the degradation of heme (Fe-protoporphyrin-IX) and its production of biliverdin and finally bilirubin (114). There are two known isoforms of heme oxygenase which exist in the diaphragm, HO-1 and HO-2 (12, 112). Of the two isoforms, HO-1 has been shown to play a critical role in redox balance involving skeletal muscle (12, 41, 109). On the contrary, there is limited evidence showing a major role for HO-2 in skeletal muscle (12). Therefore, our focus has been conserved for HO-1 on cellular redox balance in these experiments (Fig 2-2).

Various preparations including heavy metals, endotoxin, inflammatory cytokines, hydrogen peroxide (H_2O_2), quinones, and radiation (ultraviolet A) all have been shown to induce HO-1. Importantly, this provides protection where upon inhibition of HO-1 exacerbates cellular injury (3, 12, 46-48, 57, 109). The induction of HO-1 depends highly on transcriptional activation of the HO-1 gene and the synthesis of mRNA regardless of cell type. Numerous observations have concluded that pretreatment with a HO-1 inducing agent can buffer cellular stress including inflammatory and cardiovascular diseases, lung injury, ischemia/reperfusion and organ transplantation (reviewed in(91).

The protective effect of HO-1 is believed to function by the action of bilirubin where it has been shown to effectively quench singlet oxygen, scavenge hypochlorous acid, and inhibit lipid peroxidation. Bilirubin protects against cytotoxicity induced by H_2O_2 and/or enzymatically generated ROS in endothelial cells, vascular smooth muscle cells, and cardiac myocytes (19, 76, 126). In fact, Baranano et al. found that bilirubin protects against H_2O_2-mediated cell death at a 10,000 fold molar ratio of oxidant to antioxidant, suggesting even a higher buffering capacity provided by the major cellular antioxidant glutathione (10).

Recent work characterizing the gene/promoter regulation of HO-1 has uncovered additional protective roles of the enzyme's induction. These experiments describe the regulation of the antioxidant response element (ARE) which is responsible for the expression of HO-1 and other antioxidant enzymes. In particular, γ-glutamylcysteine synthetase, NAD(P)H quinone reductase, glutathione S-transferase, thioredoxin, and ferritin light/heavy chains are all regulated through ARE induction (36, 49, 123). These enzymes have all been implicated in improving the redox capacity of cells to maintain cellular homeostasis.

Contrary to the overwhelming support for HO-1 and maintenance of cellular redox control, there is also evidence for potential deleterious effects. The possibility exists where HO-1 acts as a pro-oxidant due to the release of reactive iron during the degradation of heme(57, 92). Transitional metal ions such as iron are capable of converting superoxide and H_2O_2 to the highly reactive hydroxyl radical ($^{\cdot}OH$) (shown below).

$Fe^{+2} + H_2O_2 \rightarrow$ intermediates$\rightarrow Fe^{+3} + {}^{\cdot}OH + OH^{-}$

$O_2^{\cdot -} + H_2O_2$ (with reactive iron or copper)$\rightarrow O_2 + {}^{\cdot}OH + OH^{-}$

Iron dependent radical production can function as a potent mediator of skeletal muscle injury (29, 73), Although the of and possible injury from these reactive iron exists, another positive feature of increased HO-1 is associated with the release of iron (7, 25, 26). In a variety of *in vitro* preparations the iron binding protein ferritin elicits cytoprotective properties (7, 8, 68, 122). Ferritin sequesters free intracellular iron where it can accommodate an ~ 1:4500 molar ratio(91). In this sense, the release of iron as a consequence of HO-1 activity may actually contribute to the protective mechanisms associated with HO-1 (122). The possibility of free iron mediated cellular damage via induction of HO-1 exists but is not substantially supported.

Apart from the well-defined function of HO-1 in the breakdown of heme, it is now generally accepted as a mediator of cellular protection. Applications are being explored clinically where utilization of bile pigments have been effective in decreasing organ ischemia/reperfusion injury and transplant rejection (91). Moreover, CO administration has been effective in alleviating the effects of inflammation, hypertension, organ transplantation, and vascular injury(91). Thus, the therapeutic strategies that are currently under investigation are defining the protective properties of HO-1. To date, there have been only three investigations examining the role of HO-1 in the diaphragm (12, 109, 112). These experiments proved HO-1 to be critical in

preserving diaphragmatic contractile and mitochondrial function in addition to maintaining redox buffering capacity during periods of inflammation. However, it is still unclear whether modulation of the HO-1 enzyme during MV functions in a pro- or antioxidant manner. Therefore, elucidating the role of HO-1 is of keen interest in understanding redox homeostasis of the diaphragm during ventilation.

Summary

Through extensive research, our laboratory has demonstrated the use of MV as a model for studying skeletal muscle disuse and dysfunction of the diaphragm (15, 21-23, 28, 70, 71, 86, 98-100, 118, 119, 129). By keeping an aseptic setting and maintaining blood gas homeostasis, MV serves as an excellent model for examining the mechanisms contributing to diaphragmatic injury and failure. Our lab has illustrated the effects of oxidative stress, proteolysis, and atrophy on diaphragm myofiber function during various periods of MV. However, it is still unknown which oxidant pathway(s) are responsible for these observations. Examining the role of HO-1 during MV will provide clarification of potential mechanisms causing complications in the diaphragm. Importantly, if redox balance can be maintained during MV, it is possible that rates of protease activity (calpain and caspase-3) may also be attenuated. In this regard, HO-1 may not only preserve redox status but may also limit the degree of atrophy and dysfunction of the diaphragm.

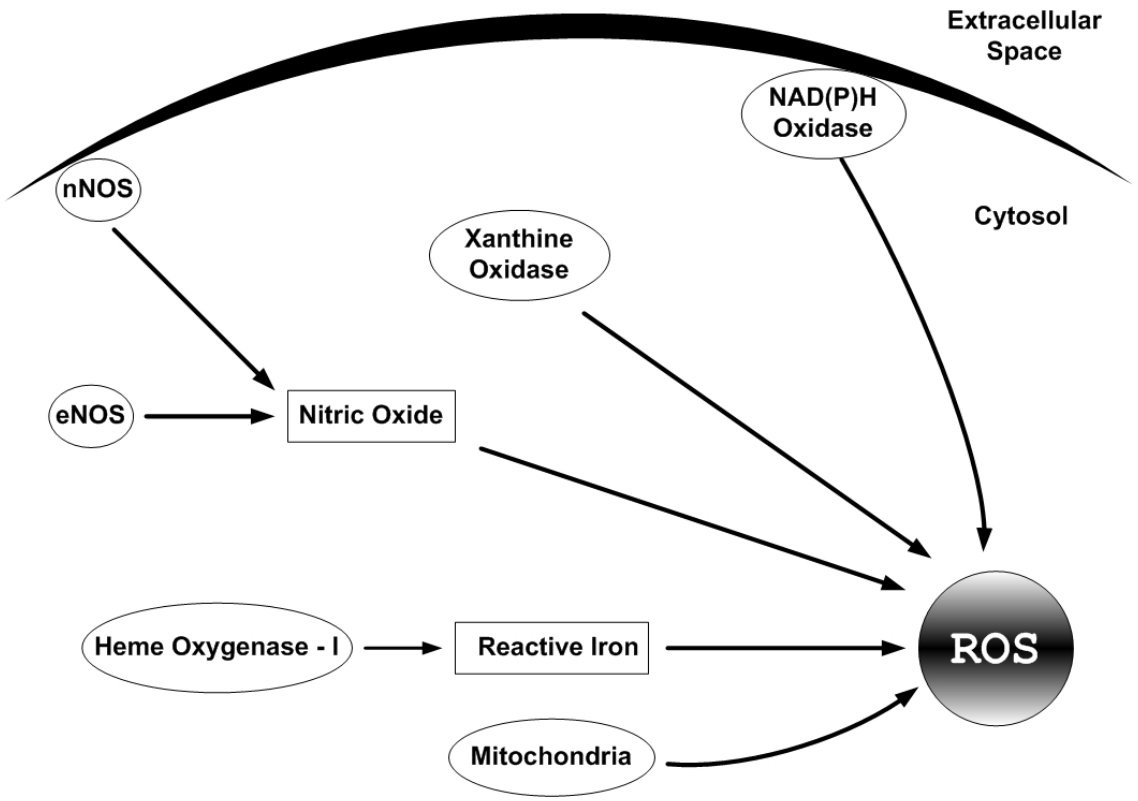

Figure 2-1. Potential sources of oxidant production in the diaphragm during MV. Periods of MV may cause an increase in one or more of the following: 1) xanthine oxidase; 2) nitric oxide synthase; 3) NADPH oxidase; 4) mitochondrial; and 5) HO-1. Through these mechanisms, MV may cause an increase in the level of oxidative stress and subsequent injury of diaphragm myofibers.

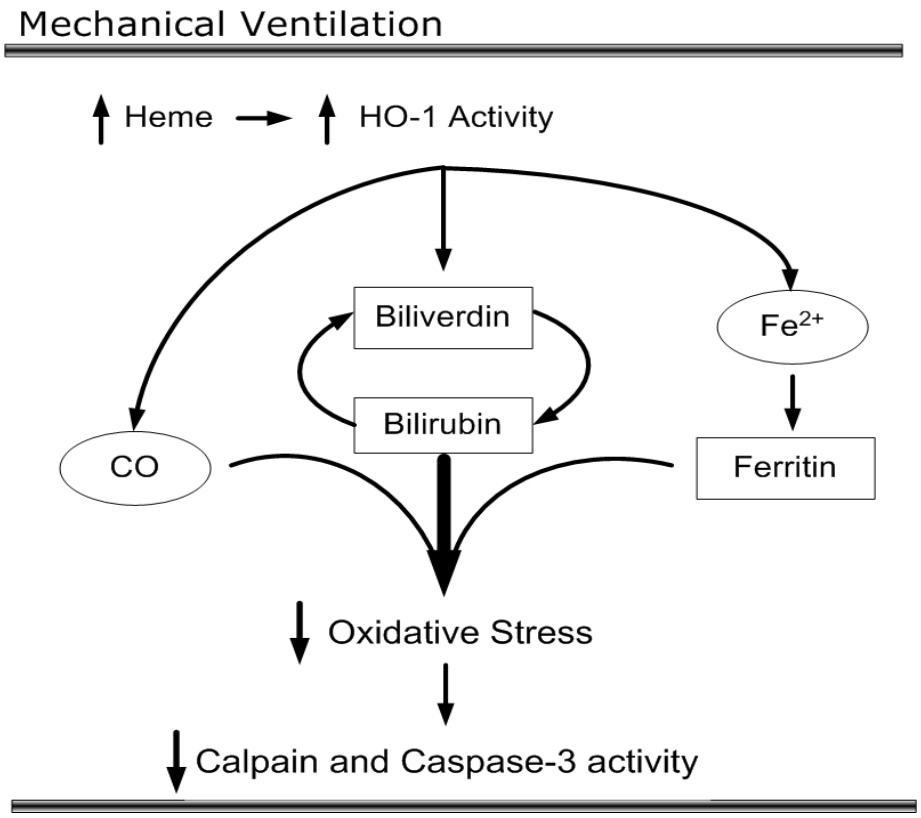

Figure 2-2. Proposed mechanism of HO-1 induced cytoprotection in the diaphragm during MV. An increase in available heme stimulates the induction of the HO-1 gene and protein thereby increasing the activity of the enzyme. Upon the degradation of heme, the by-products carbon monoxide (CO), biliverdin and iron will be released. We propose a cytoprotective effect from this action where a decrease in oxidative stress and proteolysis is observed in the diaphragm.

CHAPTER 3
MATERIALS AND METHODS

This chapter will be divided into two segments. The first segment contains the investigative designs used in each of our experiments that are intended to determine if HO-1 acts as a pro- or antioxidant in the diaphragm during MV. In a subsequent section, we will provide the methodological details associated with each experimental protocol and measurement technique.

Experiment 1: Animals

Animal Model Justification

Adult (4-6 month old) female Sprague-Dawley (SD) rats were used for experiment I. The animals were 4-6 months of age (young adult) at the time of sacrifice. The SD rat was chosen due to similarities with the human diaphragm in anatomical and physiological parameters and the size of the animal also permits periodic arterial blood sampling (5, 6, 74, 75, 82, 83, 130).

Animal Housing and Diet

All animals were housed at the University of Florida Animal Care Services Center according to guidelines set forth by the Institutional Animal Care and Use Committee. Animals were maintained on a 12:12 hour light-dark cycle and provided food (AIN93 diet) and water *ad libitum* throughout the experimental period.

Experimental Design

In experiment I, adult rats (Harlan County) were randomly assigned to one of the following groups: 1) acutely anesthetized control (n = 8), 2) 12 hours of MV (n=8), 3) 12 hours of MV with CrMPIX (n = 8), 4) 12 hours of MV with hemin (n=8), 5) 18 hours of MV (n=8), 6) 18 hours of MV with CrMPIX (n=8), and 7) 18 hours of MV with hemin (n=8) (Figure 3-1). The Animal Care and Use Committee of the University of Florida has approved these experiments.

Animal Protocol

Animals in the control group were acutely anesthetized with sodium pentobarbital (60 mg/kg IP). After reaching a surgical plane of anesthesia, the control animals were sacrificed immediately and the costal diaphragms were quickly removed and stored at –80°C for subsequent analyses.

Animals in the MV group were acutely anesthetized with sodium pentobarbital (60 mg/kg IP). After reaching a surgical plane of anesthesia, the animals were tracheostomized utilizing aseptic techniques and mechanically ventilated with a controlled pressure-driven ventilator (Seimens) for 12 or 18 hours with the following settings: upper airway pressure limit: 20 cmH_2O, pressure control level above PEEP: 4-6 cmH_2O, respiratory rate: 80 bpm; PEEP: one cmH_2O (15, 119). We have chosen 12 and 18 hours of mechanical ventilation in order to establish a time course for these measurements because these periods of MV are associated with diaphragmatic contractile dysfunction, myofiber atrophy, increased oxidative stress and rates of proteolysis.

Animals in the MVH group received an i.p. injection of hemin (50mg/kg) 24 hours prior to the initiation of MV. For the MVI group, animals received a 5μm i.p. injection of CrMPIX immediately prior to MV. Animals in the MV group received an i.p. injection of saline and surgical preparation, procedures, and animal monitoring were performed as previously described (28). Following the termination of each experimental group the animals were immediately sacrifice and the costal diaphragm was quickly removed and stored at –80°C for subsequent analyses.

Statistical Analysis

Group sample size was completed using a power analysis of preliminary data from our laboratory. Comparisons between groups were made by a 1 way ANOVA and, when appropriate, a Tukey HSD test was performed. Significance was established at $P < 0.05$.

Experiment 2: Cell Culture

The myogenic cell line was chosen due to the similarities between C2C12s and rodent skeletal muscle in cellular signaling and protein regulation (24, 33, 63-66). Cells were maintained in a temperature and gas controlled incubator throughout the experiments.

Experimental Design

In experiment II, cells were placed in one of the following groups: 1) control, 2) control/ H_2O_2, 3) hemin, 4) hemin/ H_2O_2, 5) hemin/inhibitor, and 6) hemin/inhibitor with H_2O_2 (Fig 3-2).

Cell Protocol

Myogenic cells were cultured according to Li(67). Briefly, myotubes were cultured in DMEM supplemented with 10% fetal bovine serum and gentamicin at 37ºC in the presence of 5% CO_2. Myoblast differentiation was initiated by replacing the growth medium with differentiation medium supplemented with 2% horse serum. Differentiation was allowed to continue for 96 hours before experimentation, changing to fresh media every 48 hours. At the time of harvest, myotubes were washed 3x in PBS buffer (pH 7.4) before the addition of cell lysis buffer. After addition of lysis buffer, myotubes were collected and centrifuged at 800g for 10min at 4°C. The supernatant was collected and stored at –80°C for subsequent analyses.

In the hemin group, myotubes were cultured in horse serum combined with hemin (150μM) and were incubated for 4 hours prior to harvest. Myotubes in the inhibitor group were grown in horse serum combined with CrMPIX (5μM) for 4 hours. Finally, myotubes in the

hemin/inhibitor group were grown in horse serum with hemin (150μM)/inhibitor (5μM) for 4 hours. At the time of harvest, myotubes were collected as described above.

We chose 100μM H_2O_2 for the oxidative challenge in these experiments. This concentration of H_2O_2 has been used in the literature in order to induce a stress response in C2C12 myotubes. In addition, this concentration may have relevance to *in vivo* cellular levels. Therefore, we chose this concentration to induce oxidative stress in the C2C12 protocol. H_2O_2 treatment was administered for 2 hours following incubation with groups containing saline, hemin, or hemin/inhibitor. Following incubation with H_2O_2, myotubes were collected as described above.

Statistical Analysis

Group sample size was completed using a power analysis of preliminary data from our laboratory. Comparisons between groups were made by a 1 way ANOVA and, when appropriate, a Tukey HSD test was performed. Significance was established at $P < 0.05$.

General Methods

Animal Measurements

Histological

Immunohistochemistry. Diaphragm samples were removed and fixed in OCT and stored at –80°C. On the day of analysis, diaphragm sections (8 microns) from all groups were obtained and allowed to air dry at 25°C for 30 minutes. The slides were fixed in acetone (4°C) for 5 minutes and washed in separate 2 minute washes in phosphate buffered saline. Serum blocking was performed on sections with incubation of normal goat serum (5%) blocking solution for 30 minutes. Primary HO-1 antibody incubation was diluted at 1:1000 in dystrophin solution for 60 minutes at 25°C. This was followed by an additional wash in PBS buffer with rhodamine for 30

minutes at 25°C. Secondary antibody incubation was done at a concentration of 1:100 (goat anti-mouse IgG) with Rhodamine for 30 min at 25°C. A final rinse in PBS-T (3x) at 2 minute intervals was performed on all sections. Slides were then mounted in fluorescent mounting medium (Vector Laboratories) and images were acquired via a monochrome camera (Qimaging Retiga) attached to an inverted fluorescent microscope (Axiovert 200, Xeiss).

Myofiber Cross-Sectional Area. Sections from frozen diaphragm samples were cut at 10 (microns) using a cryotome (Shandon Inc., Pittsburgh, PA) and stained for dystrophin, myosin heavy chain (MHC) I and MHC type IIa proteins for fiber cross-sectional area analysis (CSA) as described previously (71). CSA was determined using Scion software (NIH).

Biochemical

Heme Oxygenase-1 Activity. Heme Oxygenase-1 is the inducible isoform of Heme Oxygenase and catalyzes the reaction of NADPH and O_2 for oxidation of heme producing CO, ferrous iron and biliverdin. The bile pigment biliverdin is reduced to bilirubin via biliverdin reductase and both biliverdin and bilirubin are known to possess antioxidant properties (103, 117). HO activity in the microsomal fraction of diaphragm homogenate was assessed according to Gonzalez with modifications(34). Briefly, diaphragms were homogenized using a Polytron homogenizer in a 30 mM Tris-HCL buffer containing 0.25 M sucrose; 0.15 M NaCl; 1mM DTT and a commercially available protease inhibitor cocktail (Sigma, St. Louis, MO). After brief sonication, crude homogenate was centrifuged at 10,000g for 15 min at 4°C and the subsequent supernatant fraction centrifuged at 100,000g for 60 minutes at 4°C. Microsomal fractions were resuspended in 0.1 ml of 100mM potassium phosphate buffer (pH 7.4), containing 2 mM MgCl2. Protein concentration was determined using the Bradford method. HO-1 activity was assessed by combining the microsomal fraction (1 mg), biliverdin reductase (2 mg), 100mM potassium

buffer (pH 7.4), containing 2mM MgCl2, 10μM hemin, 2mM glucose 6-phosphate, 0.2 U glucose 6-phosphate dehydrogenase, and 0.8 mM NADPH. Samples were incubated for 60 minutes at 37°C and terminated by the addition of 1 mL chloroform (Sigma, St. Louis, MO). The amount of extracted bilirubin was calculated by the difference in absorption between 464nm and 530nm.

Western Blot Analysis. Protein content was determined in all samples via Western Blot analysis. Diaphragm samples were homogenized in a buffer containing: 30mMTris-HCL (pH 7.5), 250mM Sucrose, 150mM NaCl, 1mM DTT, and protease inhibitor cocktail (Sigma). Homogenates were centrifuged at 4°C for 12 minutes at 16000g. After centrifugation, the supernatant was collected and a protein assay (Bradford) was performed. Proteins from the supernatant fraction were separated via polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and incubated with primary antibodies directed against the protein of interest. 4-HNE was probed as a measurement indicative of oxidative stress while HO-1, ferritin and gamma-GCS were performed to assess redox balance. Proteolytic activity was assessed by analyzing both total (non-active) and cleaved (active) calpain I and caspase-3. In addition, calpain II, calpastatin and α-II spectrin were measured to obtain a more defined picture of calpain regulation during MV. Blots were imaged using an Odyssey system (Li-Cor Biosciences), using direct infrared fluorescent detection with a wide linear dynamic range.

Nuclear and Cytosolic Fractionation. Fractionation of diaphragm tissue was performed according to Alway et al(101). Briefly, diaphragm samples were homogenized in Buffer A containing 10mM NaCl, 15mM MgCl2 and 20 mM Hepes, Glycerol 20%, Triton X-100 0.1%, 1mM DTT and protease inhibitor cocktail (Sigma). Diaphragm homogenates were centrifuged at 880g for 3 min at 4°C. Supernatants (cytosolic fraction) were collected and the pellet was saved

for nuclear fractionation. Cytosolic preparation was performed by centrifugation of the supernatant at 3500g for 5min at 4°C and this was repeated 3 times by collecting the supernatant after each spin. Nuclear preparation was performed by washing the nuclear pellet with Buffer A and centrifugation at 880g for 3 min at 4°C. This step was repeated 3 times and pellets were the resuspended in Buffer A containing 41.5µl of 5 M NaCl. Resuspended nuclear pellets were rotated for 60 minutes at 4°C. Samples were centrifuged at 21,900g for 15min at 4°C after rotation and the final supernatant was collected, as it is the nuclear protein fraction.

Total Glutathione. Diaphragm tissue was assayed for total glutathione content using a commercially available kit according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI).

Cell Culture Measurements

Trypan Blue Exclusion Assay. The trypan blue assay is used to assess cellular viability upon exposure to experimental treatments. Briefly, an aliquot of cell suspension was diluted 1:1 with 0.4% trypan blue and cells were counted with a hemocytometer. Results are expressed as the percentage ratio of viable (unstained) cells in treated vs. untreated samples.

Western Blot Analysis. Protein content was determined in all samples via Western Blot analysis. Myotubes were lysed in a buffer containing: 30mM Tris-HCL (pH 7.5), 250mM Sucrose, 150mM NaCl, 1mM DTT, and protease inhibitor cocktail (Sigma, St. Louis). Lysates were centrifuged at 4°C for 12 minutes at 16000g. After centrifugation, the supernatant was collected and a protein assay (Bradford) was performed. Proteins from the supernatant fraction were separated via polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and incubated with primary antibodies directed against the protein of interest. 4-HNE adduct accumulation was probed as a measurement indicative of oxidative stress. HO-1 was probed to

verify HO-1 induction during the experiments. Blots were imaged using an Odyssey system (Li-Cor Biosciences), using direct infrared fluorescent detection with a wide linear dynamic range.

Total Glutathione. Diaphragm tissue was assayed for total glutathione content using a commercially available kit according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI).

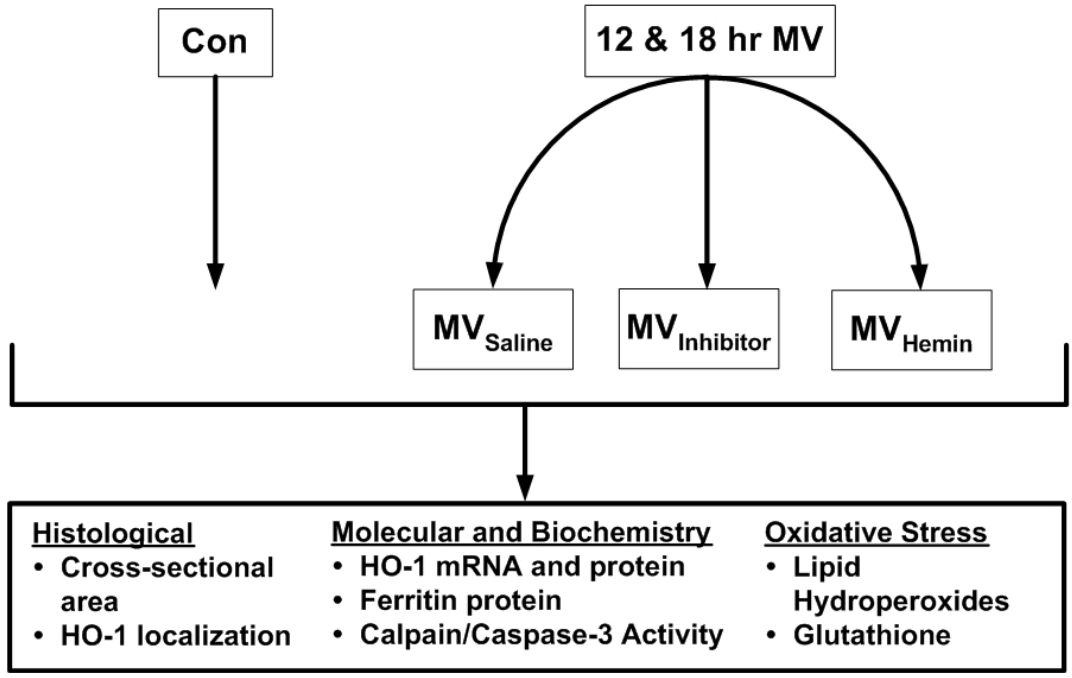

Figure 3-1. Experimental animal design examining the role of HO-1 in the diaphragm during MV. Measurements from MV, MVInhibitor, and MVHemin were made vs. CON. We addressed Aims 1 and 2 by having three levels of MV at two different durations.

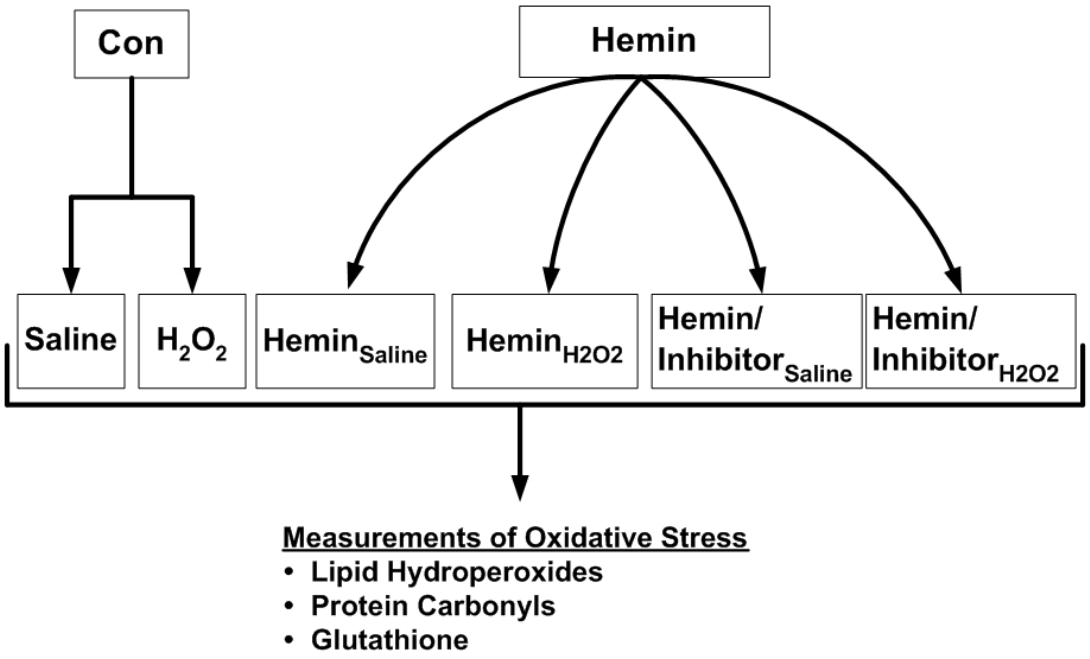

Figure 3-2. Experimental design for myogenic cell culture experiments. Treatments included saline and hydrogen peroxide for control treatments and hydrogen peroxide, HO-1 inhibitor/saline; HO-1 inhibitor/hydrogen peroxide was used in conjunction with hemin treatments in order to determine the role of HO-1 in redox regulation.

CHAPTER 4
RESULTS

Animal Characteristics

The physical and vital characteristics recorded for animals during all experiments are presented in Table 4-1. No significant differences in animal body weight, heart rate, or blood pressure existed between experimental groups.

Redox Balance

HO-1

Cellular protection mediated via HO-1 induction is thought to reside through action of bilirubin where it has been shown to effectively quench singlet oxygen, scavenge hypochlorous acid, and inhibit lipid peroxidation. Bilirubin protects against cytotoxicity induced by H_2O_2 and/or enzymatically generated ROS in endothelial cells, vascular smooth muscle cells, and cardiac myocytes. Compared to control, HO-1 protein levels in the diaphragm were significantly increased in all MV treatments (Figure 4-1). Moreover, MVH treatment resulted in a 4.5 fold further increase in diaphragmatic HO-1 levels at 12 hours and ~6 fold increase at 18 hours when compared to MV treatment alone. Thus, prolonged MV induces HO-1 protein expression in the diaphragm and when combined with the HO-1 inducer hemin, there is a synergistic effect.

Ferritin

In a variety of *in vitro* preparations the iron binding protein ferritin elicits cytoprotective properties during periods of oxidative stress. Ferritin sequesters free intracellular iron and therefore increased ferritin content in cells would attenuate the oxidant generating potential of reactive iron. In 12 and 18 hour MV experiments, both the MV and MVH treatments resulted in a significant increase in ferritin protein content in the diaphragm (Figure 4-2). In contrast, the MVI treatment did not result in a significant rise in diaphragmatic ferritin protein following 12 or

18 hours of MV. This finding could be due, at least in part, to the fact that inhibition of HO-1 activity would retard the release of free iron and therefore lower the stimulus to express ferritin.

4-HNE

4-HNE is an unsaturated α,β hydroxyalkenal that is generated during the lipid peroxidation cascade. Furthermore, 4-HNE is the primary adduct formed during this process and is commonly used to assess protein damage during oxidative stress. In 12 hour MV experiments, a significant increase in diaphragmatic 4-HNE was observed in the MV group only with no change in diaphragmatic levels of 4-HNE in the MVI group. However, in 18 hour experiments, both MV and MVI treatments result in a significant increase in 4-HNE accumulation in the diaphragm whereas diaphragmatic 4-HNE levels in the MVH animals were maintained at basal levels (Figure 4-3). Thus, it appears hemin-induced expression of HO-1 in the diaphragm assists in maintenance of redox balance during MV and attenuates the degree of oxidative cellular injury.

Total Glutathione

Glutathione is the major nonezymatic antioxidant component of the cell. It is present in mM concentrations and depletion of cellular stores is generally considered indicative of oxidative stress. Following 12 and 18 hours of MV there was a significant depletion of total GSH in diaphragms of both the MV and MVI groups. Conversely, the addition of hemin during 12 and 18 hour MV results in GSH preservation in the diaphragm (Figure 4-4).

Gamma GCS

Gamma-GCS is the rate-limiting enzyme required for glutathione synthesis. Measurements of this enzyme were performed to determine if our experimental treatments influenced the regulation of glutathione synthesis in the diaphragm. During 12 and 18hr experiments, animals in the MVH group exhibited a significant increase in gamma-GCS content when compared to Con, MV and MVI groups (Figure 4-5). Thus, maintenance of glutathione

content in MVH animals may be due, at least in part, to an increase in gamma-GCS levels in the diaphragm.

Proteolysis

Calpain I and II

The activation of the calcium activated neutral proteases, calpain I and II has been shown to occur in skeletal muscle in several models of disuse atrophy. Upon activation by elevated and sustained increases in intracellular calcium, calpains are capable of degrading both intact and disassociated skeletal muscle proteins. During 12 hour experiments (MV, MVH, and MVI), all of the experimental MV treatments resulted in a significant increase in active calpain I in the diaphragm, as detected by western blots measuring the cleaved (active) 76kDa calpain fragment (Figure 4-6). However, following 18 hours of MV, diaphragms from MVH animals do not show a significant increase in active calpain I, while calpain I activities in the diaphragms from animals in the MV and MVI treatments are still elevated. Finally, calpain II activity in the diaphragm was not altered in any groups at 12 or 18 hours when compared to control (Figure 4-7).

Caspase-3

The cleavage of procaspase-3 results in the activation of caspase-3. Once active, caspase-3 can independently participate in the breakdown of intact myofibrillar proteins. Therefore, we evaluated the activity of this protease to assess its involvement in the cleavage of myofibrillar and cytoskeletal proteins in the diaphragm during MV. Utilizing immunoblotting techniques our results indicate that in all MV treatments (MV, MVH, and MVI) diaphragmatic caspase-3 acitivity was significantly elevated at 12 and 18 hours (Figure 4-8).

Cytoskeletal Protein Degradation

α-II spectrin. α-II spectrin is a cytoskeletal structural protein present in skeletal muscle. During periods of increased proteolytic activity, α-II spectrin exhibits signature cleavage products that can be used to detect cleavage by both calpain and caspase-3. Specifically, the intact form of α-II spectrin exists as ~250kDa protein and upon degradation yields a 145 kDa (calpain specific) and 120kDa (caspase-3) cleaved bands. Thus, probing α-II spectrin cleavage products can be used to determine *in vivo* calpain and caspase-3 activities. Twelve hours of MV resulted in a significant increase in diaphragmatic levels of cleaved 120kDa α-II spectrin in the MV group (Figure 4-10). However, following 18 hours of MV, there was a significant increase in both calpain and caspase-3 specific degradation products (i.e. 145kDa and 120kDa bands, respectively) α-II spectrin in diaphragms of both MV and MVI groups (Figures 4-9 and 4-10, respectively).

Diaphragmatic Atrophy

Cross-sectional Area

To evaluate the impact of MV with overexpression and inhibition of HO-1 activity in the diaphragm, we compared the cross-sectional areas (CSA) of diaphragmatic myofibers across our experimental groups. Compared to control, we observed a significant decrease in diaphragmatic type I fiber CSA in the MV and MVI groups following 18 hours of MV. In contrast, the MVH treatment reduced diaphragmatic atrophy as evidenced by the finding that diaphragmatic type I, IIa, and IIb/x fiber CSA did not differ between control and MVH animals. Although the mean cross sectional areas of type I, IIa, and IIx/b diaphragm fibers did not differ ($P>0.05$) between the control group and the MVH group, note that no significant differences also existed in diaphragm fiber CSA between the MV group and the MVH group. Nonetheless, type I fibers were significantly larger in diaphragms from MVH treated animals compared to the MVI group

(Figure 4-11). Finally, significant decreases in CSA for type IIa and type IIb/x were observed in diaphragms from MV and MVI animals while no significant differences existed in fiber CSA between Con and MVH (Figure 4-12, 4-13).

Myogenic Cells

Cell Viability

In our cell culture experiments, we performed cell viability measurements on C2C12 myotubes treated with hemin (150μM). These measurements were performed to determine if the hemin treatment was toxic to our cell line. Importantly, our results revealed that hemin treatment was not toxic to myocytes in our experimental model as indicated by the failure of myotubes to take up Trypan blue (Figure 4-14).

HO-1 Protein

Measurements were made to assess HO-1 protein content in our cultured myotubes to determine if there was a similar induction of HO-1 in cell culture compared to the in vivo animal experiments. When compared to CON, hemin treated cells exhibited an ~15 fold increase in HO-1 protein and cells treated with hemin and hemin/inhibitor showed ~22 fold increase in HO-1 protein when compared to CON (Figure 4-15). The observation that, compared to hemin treatment alone, cells treated with both hemin and the HO-1 inhibitor exhibited significantly higher levels of HO-1 protein may be explained by a negative feedback system where the expression of the enzyme is upregulated due to the inhibition of its activity in an effort to maintain the cell's capacity to degrade heme.

Total Glutathione

Contrary to the findings in our animal experiments, cells treated with hemin did not exhibit a protective phenotype against oxidative stress when challenged with H_2O_2. In fact, hemin treated cells exposed to oxidative via H_2O_2 showed a significant decrease in total glutathione

when compared to hemin treatment alone (Figure 4-16). When comparing the experimental groups; hemin, hemin/ H_2O_2, hemin/inhibitor, and hemin/inhibitor plus H_2O_2 all showed a significant depletion of total glutathione when compared to control. Hence, these findings suggest that hemin-induced expression of HO-1 in C2C12 myotubes does not protect against H_2O_2 –induced depletion of glutathione.

4-HNE

As a second marker of oxidative stress, we also measured the levels of 4-HNE in the experimental myotubes. Identical to our aforementioned glutathione measurements in cells, compared to control, hemin treated myotubes were not protected against H_2O_2 – induced increases in cellular levels of 4-HNE (Figure 4-17). These results do not support the notion that hemin-induced increases in HO-1 levels in myotubes protects these cells against H_2O_2-induced lipid peroxidation.

Table 4-1. Body mass and vital characteristics of experimental animals. Values are expressed as mean ± SE. No significant differences were detected in any of the variables measured (P>0.05). CON= control; 12MV= 12 hours mechanical ventilation; 12MVH= 12 hours MV with hemin; 12MVI; 12 hours MV with CrMPIX; 18MV= 18 hours mechanical ventilation; 18MVH= 18 hours MV with hemin; 18MVI= 18 hours MV with CrMPIX.

Variable	**CON**	**12MV**	**12MVH**	**12MVI**	**18MV**	**18MVH**	**18MVI**
Weight (kg)	0.301±.019	0.308±.019	0.302±.029	0.292±.012	0.296±.016	0.297± .025	0.304± .014
Heart Rate (BPM)		357.6±18.4	370.4±20.0	348.3±16.4	354.1±9.2	350.7±7.9	352.9±11.1
Blood Pressure (mmHg)		96.9±14.5	101.3±12.7	97.9±9.6	76.1±11.4	79.8±6.7	83.5±21.4

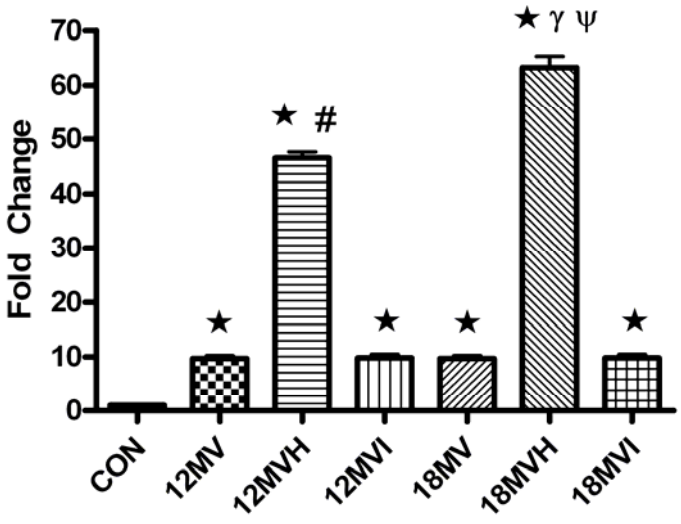

Figure 4-1. Fold changes (versus control) of HO-1 protein content in diaphragm samples. All animals mechanically ventilated exhibited a significant increase in HO-1 protein. In addition, the combination of hemin and MV resulted in an enhanced level of protein content compared to all samples measured. Y values are mean fold change ± SE. * Significantly increased versus CON ($P<0.05$). # Significantly increased versus 12MV and 12MVI ($P<0.05$). $^{\gamma}$Significantly increased versus 18MV and 18MVI ($P<0.05$). $^{\Psi}$Significantly increased versus 12MVH ($P<0.05$). CON= Control; MV= mechanically ventilated; MVH= MV with hemin; MVI= MV with CrMPIX.

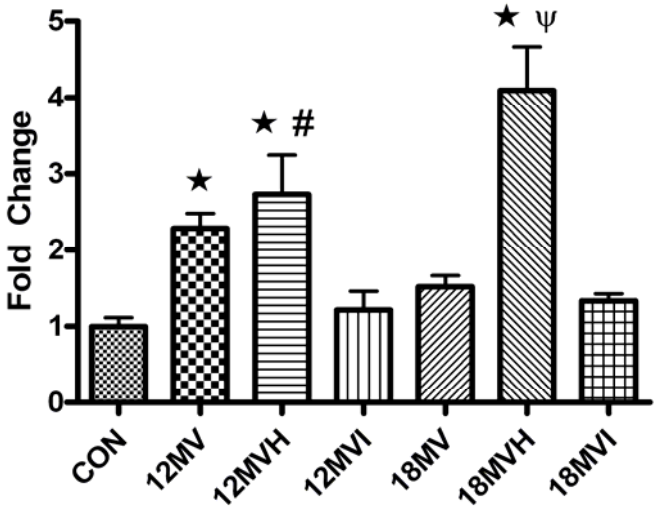

Figure 4-2. Fold changes (versus control) of ferritin protein content in diaphragm samples. MV and MVH animals exhibited a significant increase in ferritin protein. Y values are mean fold change ± SE. * Significantly increased versus Con ($P<0.05$). # Significantly increased versus 12MVI ($P<0.05$). $^{\Psi}$Significantly increased versus 18MV and 18MVI ($P<0.05$). CON= Control; MV= mechanically ventilated; MVH= MV with hemin; MVI= MV with CrMPIX.

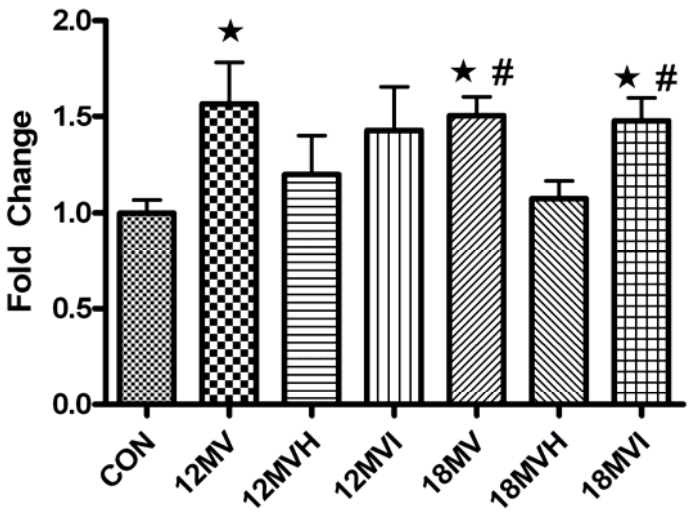

Figure 4-3. Fold changes (versus control) of 4-HNE accumulation in diaphragm samples. Y values are mean fold change ± SE. Significant increases in 4-HNE accumulation in 12MV, 18MV, and 18MVI treatments versus CON ($P>0.05$). # Significantly increased versus 18MVH ($P<0.05$). CON= Control; MV= mechanically ventilated; MVH= MV with hemin; MVI= MV with CrMPIX.

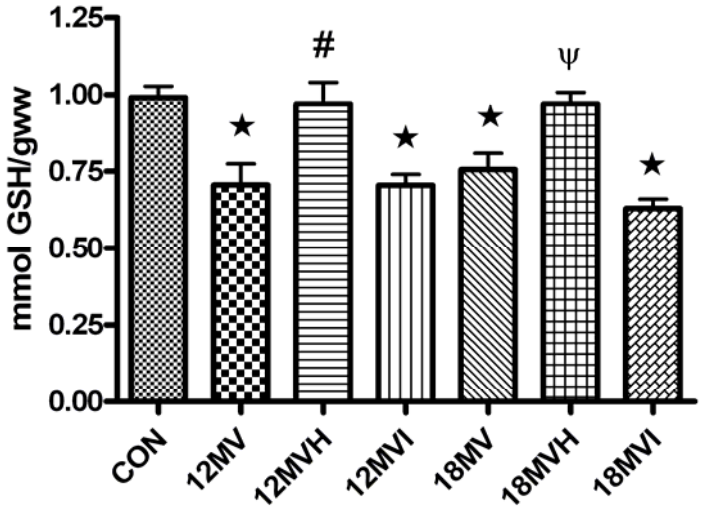

Figure 4-4. Total glutathione concentrations in diaphragm samples. 12 and 18MV and 12 and 18MVI significantly decreased versus CON. 12 and18MV and 12 and 18MVI significantly lower than MVH. Y values are mean ± SE. * Significantly decreased versus Con. #Significantly increased versus 12MV and 12MVI ($P<0.05$). $^{\Psi}$Significantly increased versus 18MV and 18MVI ($P<0.05$). CON= Control; MV= mechanically ventilated; MVH= MV with hemin; MVI= MV with CrMPIX.

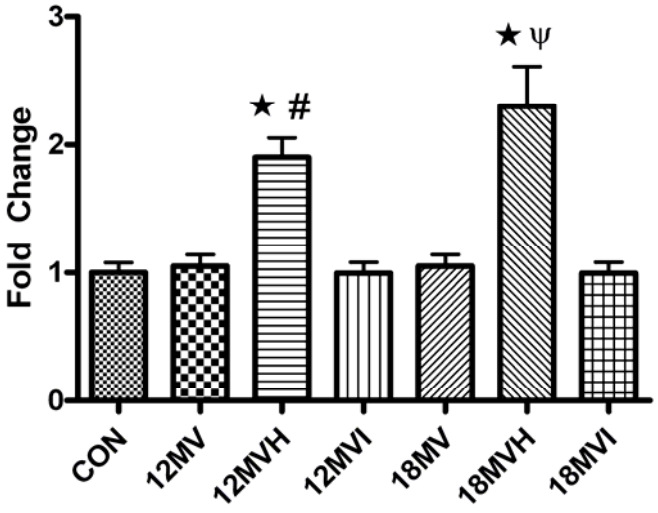

Figure 4-5. Fold changes (versus control) of gamma-GCS levels in diaphragm samples. MVH groups significantly increased versus all groups. Y values are mean fold change ± SE. * Significantly increased versus CON ($P<0.05$). #Significantly increased versus 12MV and 12MVI ($P<0.05$). $^{\psi}$Significantly increased versus 18MV and 18MVI ($P<0.05$). CON= Control; MV= mechanically ventilated; MVH= MV with hemin; MVI= MV with CrMPIX.

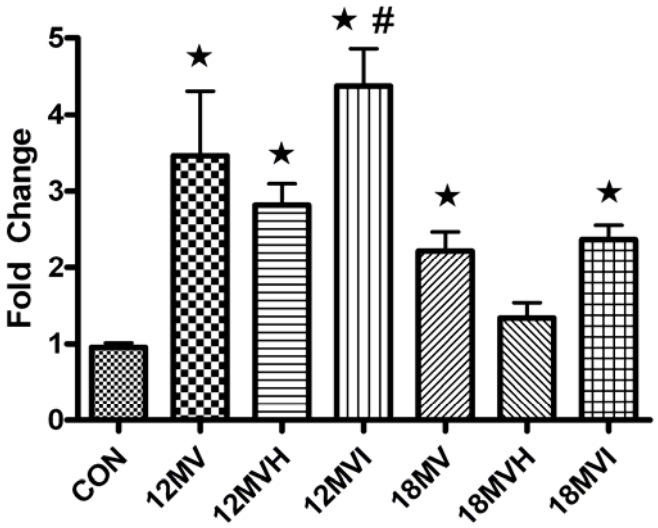

Figure 4-6. Fold changes (versus control) of active calpain I protein in diaphragm samples. 12 and 18 MV, 12MVH, and 12 and 18MVI significantly increased versus CON. Y values are mean fold change ± SE. * Significantly increased versus CON ($P<0.05$). # 12MVI significantly increased versus 18MVI ($P<0.05$). CON= Control; MV= mechanically ventilated; MVH= MV with hemin; MVI= MV with CrMPIX.

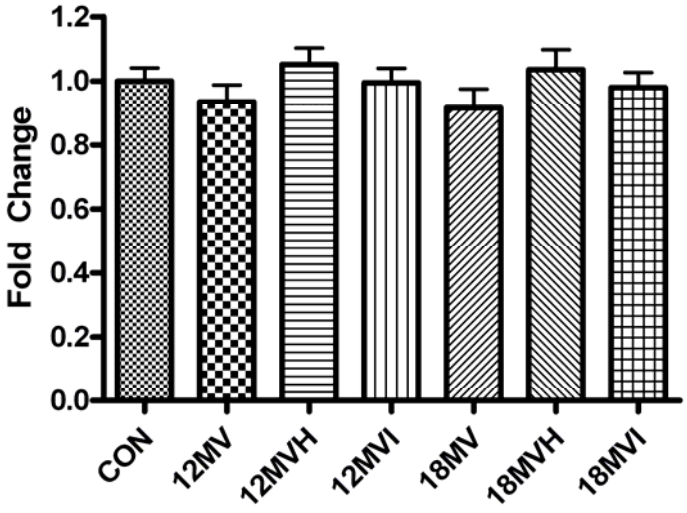

Figure 4-7. Fold changes (versus control) of active calpain II protein measurements in diaphragm samples. Y values are mean fold change ± SE. No significant difference versus Con. (P>0.05). CON= Control; MV= mechanically ventilated; MVH= MV with hemin; MVI= MV with CrMPIX.

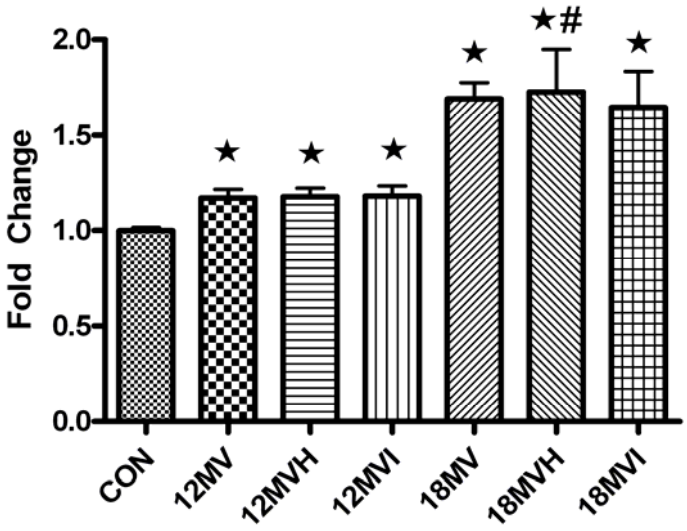

Figure 4-8. Fold changes (versus control) of active caspase-3 protein measurements in diaphragm samples. 12 and 18 hour MV, MVH, and MVI significantly increased versus CON. Y values are mean fold change ± SE. * Significantly increased versus CON (P<0.05). # Significantly increased versus 12MVH (P<0.05). CON= Control; MV= mechanically ventilated; MVH= MV with hemin; MVI= MV with CrMPIX.

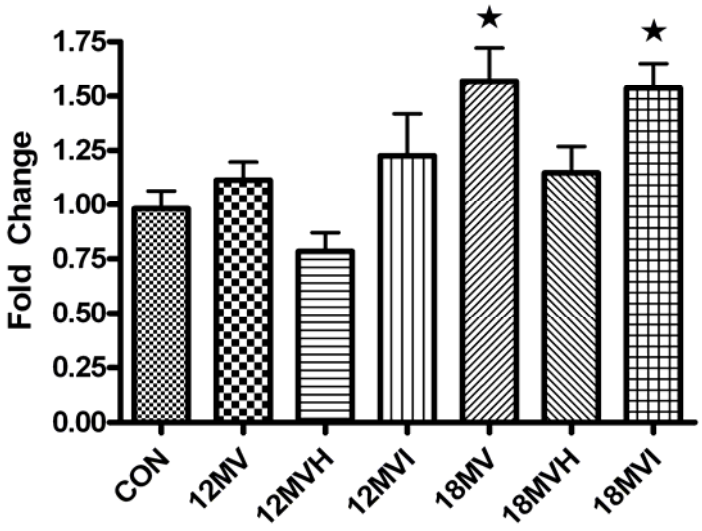

Figure 4-9. Fold changes (versus control) of 145kDa α-II spectrin degradation product levels in diaphragm samples. Y values are mean fold change ± SE. *18MV and 18MVI significantly increased versus CON ($P<0.05$). CON= Control; MV= mechanically ventilated; MVH= MV with hemin; MVI= MV with CrMPIX.

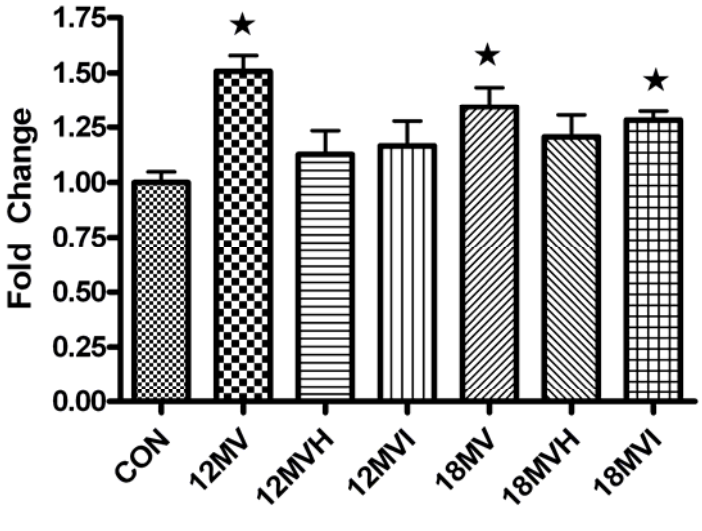

Figure 4-10. Fold changes (versus control) of 120kDa α-II spectrin degradation product levels in diaphragm samples. Y values are mean fold change ± SE. *12MV, 18MV and 18MVI significantly increased versus CON ($P<0.05$). CON= Control; MV= mechanically ventilated; MVH= MV with hemin; MVI= MV with CrMPIX.

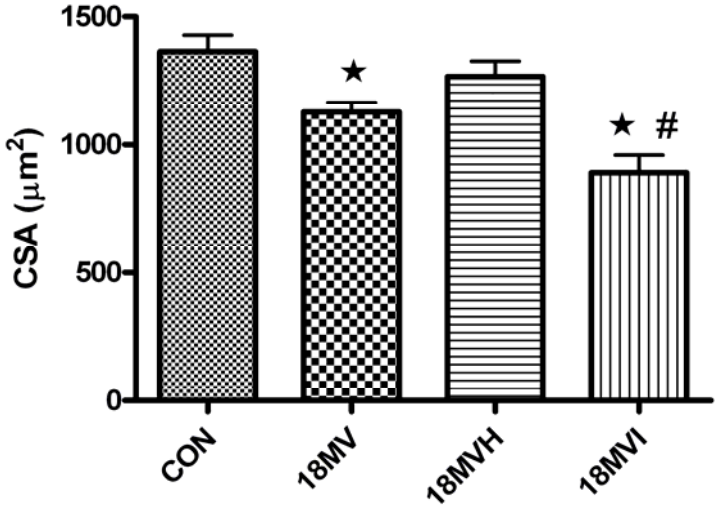

Figure 4-11. Type I fiber cross-sectional area in the diaphragm following 18 hours of MV. MV and MVI significantly decreased versus CON and MVH. MVI significantly lower than MVH. Y values are mean ± SE. * Significantly decreased versus CON. # Significantly decreased versus MVH ($P<0.05$). CON= Control; MV= mechanically ventilated; MVH= MV with hemin; MVI= MV with CrMPIX.

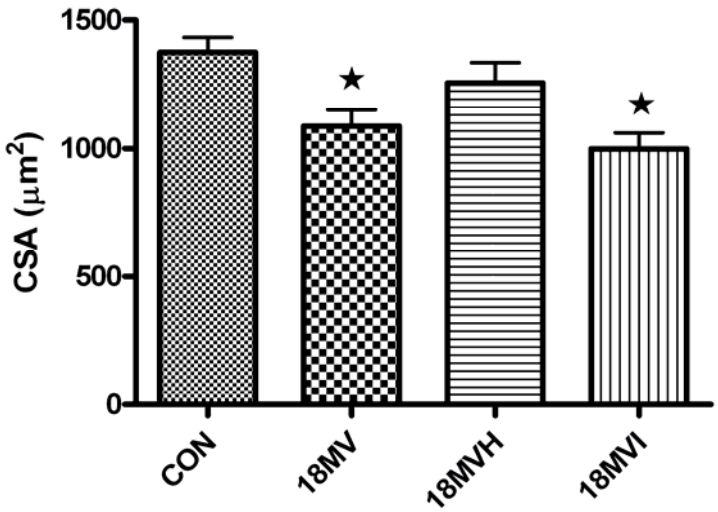

Figure 4-12. Type IIa fiber cross-sectional area in the diaphragm following 18 hours of MV. MV, MVI significantly decreased versus CON and MVH. Y values are mean ± SE. * Significantly decreased versus CON ($P<0.05$). CON= Control; MV= mechanically ventilated; MVH= MV with hemin; MVI= MV with CrMPIX.

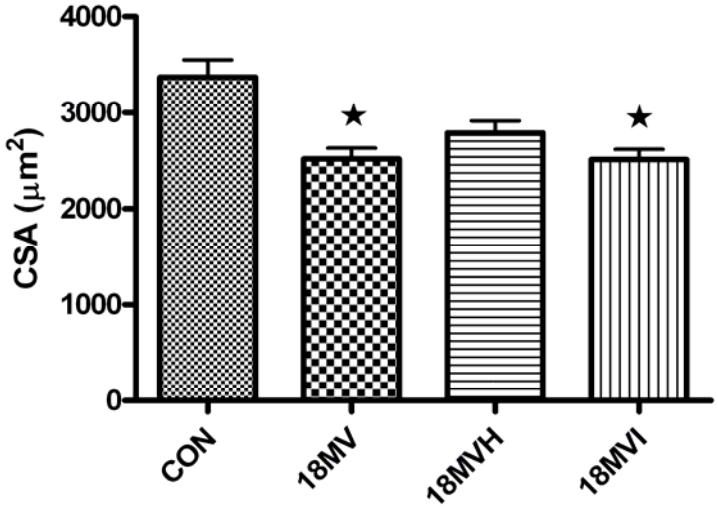

Figure 4-13. Type IIb/x fiber cross-sectional area in the diaphragm following 18 hours of MV. MV, MVI significantly decreased versus CON and MVH. Y values are mean ± SE. * Significantly decreased versus CON ($P<0.05$). CON= Control; MV= mechanically ventilated; MVH= MV with hemin; MVI= MV with CrMPIX.

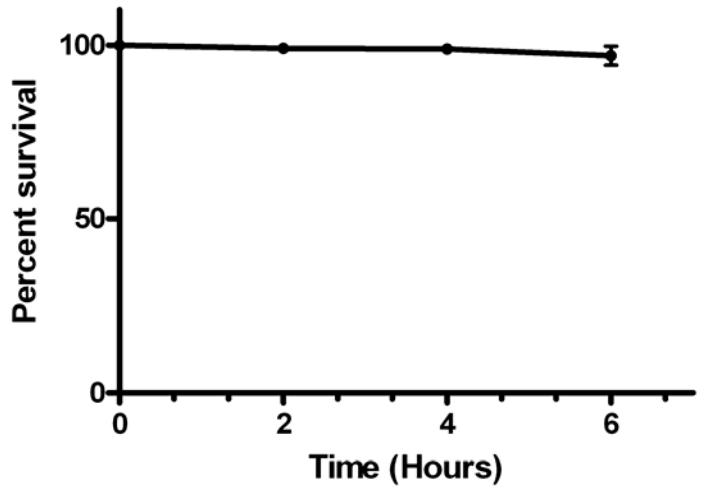

Figure 4-14. Cell viability after treatment with hemin (150μM). Cells were treated with hemin and viability was assessed using the Trypan blue exclusion method at 0, 2, 4 and 6 hours following treatment. Y values are mean percent survival ± SE. No significant difference existed between control and any time period (P>0.05).

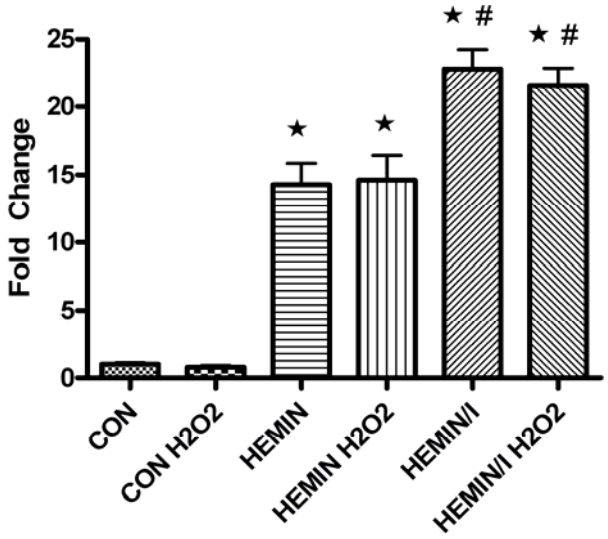

Figure 4-15. Fold change (versus control) of HO-1 protein levels in C2C12 myotubes. Y values are mean fold change ± SE. * Significantly increased versus CON and CON H2O2. # Significantly increased versus Hemin and Hemin H2O2 (P<0.05). CON= Control; CON H2O2= Control with H_2O_2; Hemin= Hemin; Hemin H2O2= Hemin with H_2O_2; Hemin/I= hemin with CrMPIX; Hemin/I H2O2= Hemin/CrMPIX with H_2O_2.

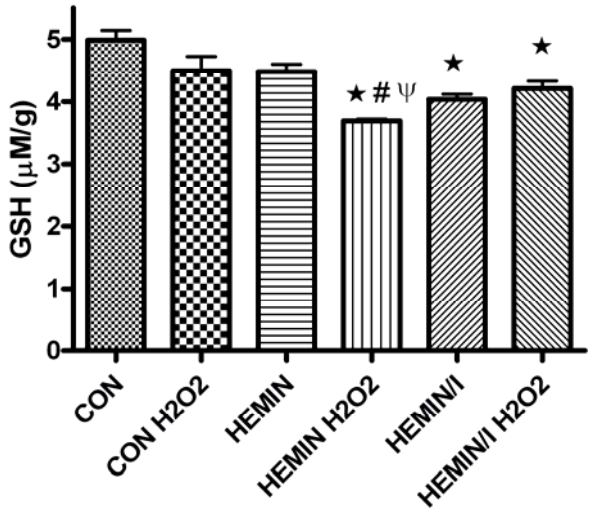

Figure 4-16. Total GSH concentration in cultured C2C12 myotubes. Y values are mean ± SE. *Hemin H2O2, Hemin/I and Hemin/I H2O2 significantly lower versus CON. #Hemin H2O2 significantly lower than CON H2O2. ψHemin H2O2 significantly lower versus H ($P<0.05$). CON= Control; CON H2O2= Control with H_2O_2; Hemin= Hemin; Hemin H2O2= Hemin with H_2O_2; Hemin/I= hemin with CrMPIX; Hemin/I H2O2= Hemin/CrMPIX with H_2O_2.

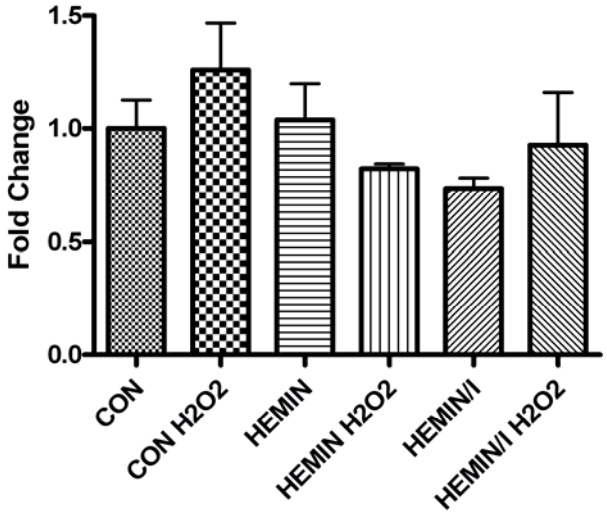

Figure 4-17. Fold change (versus control) of total 4-HNE measurements in cultured C2C12 myotubes. Y values are mean ± SE. No significant differences detected in groups ($P>0.05$). CON= Control; CON H2O2= Control with H_2O_2; Hemin= Hemin; Hemin H2O2= Hemin with H_2O_2; Hemin/I= Hemin with CrMPIX; Hemin/I H2O2= Hemin/CrMPIX with H_2O_2.

CHAPTER 5
DISCUSSION

Overview of Principal Findings

These experiments tested the hypothesis that increased levels of HO-1, an endogenous enzyme with antioxidative properties, would retard MV-induced oxidative injury in the diaphragm. Our findings support this postulate as overexpression of HO-1 attenuated MV-induced oxidative stress in the diaphragm. We also predicted that MV-induced protease activation and atrophy of the diaphragm would be diminished with elevated levels of HO-1. Our results also support this postulate as overexpression of HO-1 in the diaphragm retarded MV-induced calpain and caspase-3 activity and myofiber atrophy in type I, IIa and type IIb/x fibers.

Finally, to further investigate the antioxidant role of HO-1, we utilized a myogenic cell culture model to determine whether overexpression of HO-1 protects against ROS-mediated oxidative injury. Our results indicate that hemin-induced overexpression of HO-1 in myotubes does not protect these cells against oxidative injury induced by exposure to H_2O_2. A detailed discussion providing an interpretation of our experiments follows in the subsequent sections.

Induction of HO-1 during MV Attenuates Diaphragmatic Oxidative Stress

Prolonged MV is associated with a shift in the pro-to-antioxidant balance in the diaphragm resulting in increased protein oxidation and lipid peroxidation in this key inspiratory muscle (120). The diaphragmatic endogenous antioxidant system is intricate and involves both enzymatic and nonenzymatic antioxidants. In this regard, prolonged MV depletes diaphragmatic glutathione stores but also increases the expression of at least two antioxidant enzymes, thioredoxin reductase-1 and manganese superoxide dismutase (28). Of further interest is that HO-1 protein abundance and gene expression rapidly increase in the diaphragm during MV (28). However, there is uncertainty in the literature whether overexpression of HO-1 is beneficial or

deleterious to cells due to its reported actions as either a pro- or antioxidant (92). In theory, HO-1 can serve as a pro-oxidant by increasing the availability of free iron in the cell or as an antioxidant by producing both bilirubin and biliverdin. In an attempt to determine whether HO-1 functions as a pro- or antioxidant in the diaphragm during MV, we independently elevated HO-1 levels in the diaphragm and in parallel experiments we suppressed the activity of HO-1. Our findings support the concept that HO-1 acts as an antioxidant in the diaphragm as hemin-induced overexpression of HO-1 was associated with reduced oxidative stress during MV as indicated by the preservation of glutathione levels and the attenuation of lipid peroxidation. Moreover, pharmacological inhibition of HO-1 activity was associated with the normal progression of MV-induced oxidative stress in the diaphragm, verifying that HO-1 does not act as a pro-oxidant in this model.

Overexpression of HO-1 Reduces Overall MV-induced Protease Activation

It is well established that inactivity in skeletal muscles results in the activation of proteolytic pathways and that antioxidant administration has been successful in decreasing proteolysis (15, 72, 97). Therefore, we postulated that if HO-1 acts as an antioxidant in skeletal muscle, overexpression of HO-1 would diminish the activation of key proteases (i.e., calpain and caspase-3) in the diaphragm during prolonged MV. We focused our interest on calpain and caspase-3 because the induction of the calpains (i.e. I and II) and caspase-3 during periods of disuse may be a required first step to initiate the cascade of events underlying skeletal muscle atrophy (85, 111, 116). Indeed, our laboratory has shown that caspase-3 plays a critical role in skeletal muscle atrophy as pharmacological inhibition of caspase-3 activity retards diaphragmatic atrophy during 12 hours of MV (72). Furthermore, the administration of a calpain inhibitor (leupeptin) is also effective in attenuating diaphragmatic atrophy and contractile dysfunction during prolonged (i.e., 24 hours) MV (69). In the present study, we assessed the influence of

HO-1 levels on protease activation in two different ways. First, we assayed the activity of both calpain (I & II) and caspase-3 via western blotting to determine the level of activation in the diaphragm at the completion of our MV experiments. Because this technique does not evaluate the contributions of calpain and capase-3 to muscle proteolysis *in vivo,* we also used an innovative and complimentary technique to assess calpain and caspase-3 activation in the diaphragm. This second technique is a novel method capable of measuring calpain and caspase-3 protease activity over time in muscle fibers. Briefly, this technique measures degradation of "specific" endogenous calpain and caspase-3 substrates (i.e., α-II spectrin) within muscle and provides an index of *in vivo* calpain and caspase-3 activity in the diaphragm over prolonged periods of MV.

Using the direct approach measuring calpain I activity, our results reveal that overexpression of HO-1 is associated with reduced calpain I activation in the diaphragm of MV animals. In contrast, overexpression of HO-1 did not appear to influence diaphragmatic caspase-3 activity at the completion of 12 or 18 hours of MV. Note, however, that these activity assays are single measurements (i.e. snapshot) of calpain and caspase-3 activity at one point in time (i.e., end of experiment). Therefore, these measures fail to provide an accurate assessment of *in vivo* activity levels in the diaphragm over time. A more valid measurement of calpain and caspase-3 activity during MV may be achieved by assessment of specific calpain and caspase-3 degradation products of α-II spectrin. Indeed, the accumulation of signature cleavage products of α-II spectrin represent the specific activity of both calpain and caspase-3. In hemin-treated animals overexpressing HO-1, a reduction in the appearance of calpain specific (145kDa) and caspase-3 specific (120kDa) cleavage of α-II spectrin breakdown products were observed in the diaphragm following 18 hours of MV. These observations suggest that HO-1 overexpression is

associated with a decrease in the proteolytic-mediated damage to structural components within the diaphragm during MV. These findings, in conjunction with previous results from our laboratory, suggest that increasing the diaphragmatic antioxidant capacity attenuates protease activation and may be effective in the preservation of diaphragmatic properties (i.e. atrophy and contractile dysfunction) during MV (15, 72).

Overexpression of HO-1 Retards MV-induced Diaphragmatic Atrophy

Based upon our finding that overexpression of HO-1 was associated with decreased oxidative stress and attenuated protease activation in the diaphragm, we anticipated that hemin-induced expression of HO-1 would significantly retard MV-induced diaphragmatic myofiber atrophy. Our results support this notion as hemin-induced expression of HO-1 protected against MV-induced atrophy in type I, type IIa, and type IIb/x fibers. Nonetheless, although the mean CSA of type I, IIa, and IIb/x diaphragm fibers did not differ ($P>0.05$) between the control group and the hemin-treated MV group, no differences also existed in diaphragm fiber CSA between the MV group and the hemin-treated MV group. We interpret this finding as an indication that while the overexpression of HO-1 diminished MV-induced myofiber atrophy, elevated levels of HO-1 did not completely prevent MV-induced diaphragmatic fiber atrophy.

Induction of HO-1 in Myotubes Fails to Maintain Redox Balance during H_2O_2 Exposure

Oxidant challenges (H_2O_2) to C2C12 myotubes can induce oxidative stress, proteolysis and myotube atrophy (65, 105). Therefore, as a means of providing a controlled *in vitro* environment to study the antioxidant potential of HO-1 to protect cells against a H_2O_2 challenge, we included a myogenic cell culture model in our experiments. Specifically, we tested the hypothesis that hemin induced increases in HO-1 levels in myotubes would provide antioxidant protection against an oxidative insult (100μM H_2O_2). Our results indicate that hemin induced an increase in HO-1 protein expression and did not alter myotube viability. However, hemin-induced

overexpression of HO-1 did not protect myotubes against H_2O_2-mediated oxidative stress (i.e., protection against lipid peroxidation). Therefore, these results differ from our *in vivo* results where overexpression of HO-1 protected the diaphragm against MV-induced oxidative damage.

Several possibilities exist for these divergent findings. First, although H_2O_2 is widely used to impose an oxidative stress-like environment in cell culture, using H_2O_2 alone does not simulate the normal *in vivo* environment where numerous radicals and reactive oxygen species (i.e. superoxide, nitric oxide, hydroxyl radicals, etc) are continually produced within skeletal muscle (4). Hence, it is possible that HO-1 possesses antioxidant scavenging properties against reactive oxygen species other than H_2O_2.

Another explanation for our divergent findings involves the complexity of determining both a physiologically relevant concentration of H_2O_2 and duration of exposure to provide an oxidative challenge in myotubes. Although intracellular concentrations of H_2O_2 have been estimated to be ≤ 1 μm in cells not experiencing oxidative stress (18), previous investigators have exposed cells to a wide range of H_2O_2 concentrations (25μm - 1mM) (65, 80, 90, 96, 128),. This disparity creates difficulty in determining the most physiologic and relevant conditions for an oxidant challenge. In our *in vitro* experiments, we exposed myotubes to H_2O_2 concentrations of 100μM and below because higher concentrations of H_2O_2 diminish cellular viability (unpublished observations). Therefore, we reasoned that it was appropriate to expose myotubes to H_2O_2 concentrations that would induce oxidative stress without decreasing cellular viability. Nonetheless, it is possible that our exposure of myotubes to exogenous H_2O_2 resulted in intracellular levels of H_2O_2 that surpass the levels observed in diaphragm fibers during prolonged MV. That is, it is feasible that the H_2O_2 levels used in our experiments overwhelmed the antioxidant properties of HO-1 and the other endogenous antioxidants. Moreover, although H_2O_2

can pass freely into the cell, concentrations within cellular compartments in *in vitro* experiments do not necessarily reflect those found in *in vivo* conditions (18). Finally, the PO_2 of the environment (95% O_2 and PO_2~677 mmHg) commonly used during the growth and differentiation of myotubes is much higher than *in vivo* levels and may result in a myotube phenotype that differs markedly from skeletal muscle fibers derived from animals. Therefore, collectively, these concerns raise important questions regarding the physiological relevance of *in vitro* myotube models of oxidative stress.

Conclusions and Future Directions

This study provides the first *in vivo* evidence that overexpression of HO-1 functions as an antioxidant in the diaphragm during MV. Specifically, these results demonstrate that HO-1 improves resistance to MV-induced oxidative stress. Furthermore, the induction of HO-1 in the diaphragm is effective in diminishing MV-induced protease (e.g., calpain and caspase-3) activation and is associated with a reduction in MV-induced diaphragmatic atrophy. Collectively, these are novel and important findings. Indeed, prior to the current experiments, the question of whether HO-1 functions as an antioxidant or pro-oxidant remained unanswered. Our results clearly indicate that overexpression of HO-1 provides protection against inactivity-induced oxidative stress in skeletal muscle fibers. Moreover, our findings suggest that pharmacological induction of HO-1 expression could be a potential therapeutic strategy to retard MV-induced oxidative stress and atrophy in the diaphragm during prolonged MV. This is clinically significant because MV-induced diaphragmatic weakness plays an important role in weaning difficulties following MV.

Future studies should focus on elucidating the pathways responsible for oxidant production in the diaphragm during prolonged MV and on the mechanism(s) that are accountable for oxidative stress-induced activation of both calpain and caspase-3. A complete understanding of

these two important issues is essential to develop effective and safe countermeasures for preventing respiratory muscle atrophy and weakness during prolonged MV. Development of a clinically useful countermeasure to prevent or retard MV-induced diaphragmatic atrophy and weakness would potentially maintain normal inspiratory muscle function during prolonged MV, which could permit patients to wean from the ventilator more successfully.

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BIOGRAPHICAL SKETCH

Darin J. Falk was born in Columbus, Nebraska. He attained a Bachelor of Science degree from the University of Nebraska-Kearney. Following graduation, he pursued a Master's degree in exercise physiology and graduated from University of Nebraska-Kearney in 2004. Deciding to focus his career in basic science, Darin began his doctoral work at the University of Florida in 2002 under the direction of Scott K. Powers. Darin focused his studies on the mechanisms underlying diaphragmatic atrophy and dysfunction induced by mechanical ventilation. He received his PhD in 2007.