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NF- κ B Signaling Contributes to Mechanical Ventilation-Induced Diaphragm Weakness

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Abstract

Background—Although mechanical ventilation (MV) is a life-saving measure for patients in respiratory failure, prolonged MV results in diaphragmatic weakness due to fiber atrophy and contractile dysfunction. Therefore, identifying the signaling pathways responsible for MV-induced diaphragmatic weakness is important. In this context, it is established that oxidative stress is required for MV-induced diaphragmatic weakness to occur. Numerous redox-sensitive signaling pathways exist in muscle including the transcription factor nuclear factor- κ B (NF- κ B). Although it has been suggested that NF- κ B contributes to proteolytic signaling in inactivity-induced atrophy in locomotor muscles, the role that NF- κ B plays in MV-induced diaphragmatic weakness is unknown.

Objective—We tested the hypothesis that NF- κ B activation plays a key signaling role in MV-induced diaphragmatic weakness and that oxidative stress is required for NF- κ B activation.

Design—Cause and effect was determined by independently treating mechanically ventilated animals with either a specific NF- κ B inhibitor (SN50) or a clinically relevant antioxidant (curcumin).

Measurements and Main Results—Inhibition of NF- κ B activity partially attenuated both MV-induced diaphragmatic atrophy and contractile dysfunction. Further, treatment with the antioxidant curcumin prevented MV-induced activation of NF- κ B in the diaphragm and rescued the diaphragm from both MV-induced atrophy and contractile dysfunction.

Conclusions—Collectively, these findings support the hypothesis that NF- κ B activation plays a significant signaling role in MV-induced diaphragmatic weakness and that oxidative stress is an upstream activator of NF- κ B. Finally, our results suggest that prevention of MV-induced oxidative stress in the diaphragm could be a useful clinical strategy to prevent or delay MV-induced diaphragmatic weakness.

Keywords

respiratory muscles; oxidative stress; atrophy; antioxidants; curcumin; weaning

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INTRODUCTION

Mechanical ventilation (MV) is used clinically to provide sufficient alveolar ventilation in patients incapable of maintaining adequate pulmonary gas exchange. For patients with acute respiratory failure, MV is a life-saving intervention. Unfortunately, MV results in a rapid development of respiratory muscle weakness which is predicted to contribute to weaning problems from MV (1, 2). Weaning difficulties are a dilemma in critical care medicine as 20–30% of mechanically ventilated patients experience weaning problems (3). Therefore, understanding the mechanism(s) leading to MV-induced diaphragmatic weakness is important.

In reference to cell signaling pathways that lead to MV-induced diaphragm weakness, it is clear that MV results in the rapid development of diaphragmatic oxidative stress (4–6). Indeed, avoidance of MV-induced oxidative damage via antioxidants can protect the diaphragm from MV-induced atrophy and contractile dysfunction (6–8). Specifically, our work reveals that oxidative stress is required for the activation of several key proteolytic systems involved in MV-induced diaphragmatic atrophy (e.g. calpain and caspase-3) (7, 9). These findings indicate that the MV-induced production of reactive oxygen species (ROS) in the diaphragm is an essential upstream trigger for signaling events that lead to diaphragm weakness.

In regard to specific redox sensitive signaling pathways that contribute to locomotor skeletal muscle proteolysis, the transcriptional activating factor nuclear factor- κ B (NF- κ B) has been suggested to play a significant role. Specifically, the NF- κ B signaling pathway promotes the expression of a key protein associated with the ubiquitin-protease system of proteolysis (i.e., E3 ligase, muscle ring finger-1) and NF- κ B activation is an important contributor to disuse-induced atrophy in locomotor skeletal muscles (reviewed in (10, 11)). Activation of NF- κ B is achieved through nuclear transport of dimers of NF- κ B family members and often occurs by the ubiquitination and degradation of the inhibitory protein I κ B, which otherwise binds NF- κ B heterodimers and retains their cytosolic residence (10, 11). Although evidence indicates that NF- κ B contributes to locomotor muscle catabolism during disuse, the role that NF- κ B plays in MV-induced diaphragmatic atrophy remains unknown. Therefore, these experiments determined if NF- κ B activation is required for MV-induced diaphragm atrophy/contractile dysfunction. We also established if oxidative stress is essential for MV-induced activation of NF- κ B in the diaphragm. Based upon our preliminary results, we hypothesized that NF- κ B activation contributes to MV-induced diaphragm atrophy and contractile dysfunction and that oxidative stress is essential for NF- κ B activation in the diaphragm during prolonged MV.

METHODS

Animals and Experimental Design

Young adult (~4–6 month old) female Sprague-Dawley (SD) rats were randomly assigned to one of four experimental groups (n = 8/group): 1) acutely anesthetized control (CON), 2) 12 hours of controlled mechanical ventilation (12MV), 3) 12 hours of MV-treated with the NF- κ B specific inhibitor SN50 (12MV-SN50), and 4) 12 hours of MV-treated with the antioxidant curcumin (12MVC). The Institutional Animal Care and Use Committee of the University of Florida approved these experiments.

Experimental Protocol

Mechanical Ventilation—All surgical procedures were performed using aseptic techniques. Animals in the MV groups were anesthetized with an IP injection of sodium pentobarbital (60 mg/kg body weight), tracheostomized, and mechanically ventilated with a pressure-controlled ventilator (Servo Ventilator 300; Siemens, Munich, Germany) for 12 hours with the following settings: upper airway pressure limit: 10 cmH₂O; and respiratory rate: 80 bpm; PEEP: 1 cmH₂O.

The carotid artery was cannulated to permit the continuous measurement of blood pressure and the collection of blood during the protocol. Arterial blood samples (100 µl per sample) were removed periodically and analyzed for arterial pO₂, pCO₂ and pH using an electronic blood-gas analyzer (GEM Premier 3000; Instrumentation Laboratory, Lexington, MA). Ventilator adjustments were made if arterial PCO₂ exceeded 40 mm Hg. Moreover, arterial PO₂ was maintained > 60 mmHg throughout the experiment by increasing the F_IO₂ (22–26% oxygen).

A venous catheter was inserted into the jugular vein for continuous infusion of sodium pentobarbital (~10 mg/kg/hr). Body temperature was maintained at 37°C by use of a recirculating heating blanket and heart rate was monitored via a lead II electrocardiograph. Continuous care during the MV protocol included lubricating the eyes, expressing the bladder, removing airway mucus, rotating the animal, and passively moving the limbs. Animals also received an intramuscular injection of glycopyrrolate (0.04 mg/kg) every two hours during MV to reduce airway secretions. Upon completion of MV, the diaphragm was quickly removed and a strip of the medial costal diaphragm was used for *in vitro* contractile measurements, a section was stored for histochemical analyses, and the remaining portion was frozen in liquid nitrogen and stored at –80°C for subsequent analyses.

SN50 Administration—The p50 inhibitor, SN50 was administered at 10 µg/kg (Calbiochem, San Diego, CA) 12 hours prior to surgery (12, 13). This dose was chosen based on previous animal studies showing that *in vivo* administration of SN50 at this dose inhibits NF- B activity by blocking the nuclear localization sequence on p50 (12, 13).

Curcumin Administration—The antioxidant curcumin (Cayman, Ann Arbor, MI) was administered using three intraperitoneal doses (600 mg/kg) one 24 hours prior to surgery, one 12 hours prior to surgery and one immediately prior to surgery. This specific dose was chosen based on previous studies demonstrating the effectiveness of curcumin administration in blocking NF- B by scavenging reactive oxygen intermediates that can activate NF- B over a range of doses (14).

Biochemical Measures

Western Blot Analysis—Protein abundance was determined in diaphragm samples via Western Blot analysis. Briefly, diaphragm tissue samples were homogenized 1:10 (wt/vol) in 5 mM Tris (pH 7.5) and 5 mM EDTA (pH 8.0) with a protease inhibitor cocktail (Sigma, St. Louis, MO) and centrifuged at 1500 g for 10 min at 4°C. After collection of the resulting supernatant, diaphragmatic protein content was assessed by the method of Bradford (Sigma). Proteins from the supernatant fraction were separated via polyacrylamide gel electrophoresis via 4–20% gradient polyacrylamide gels containing 0.1% sodium dodecyl sulfate for ~1 h at 200 V. Non-specific sites were blocked for 2 hours at room temperature in phosphate-buffered saline (PBS) solution containing 0.05% Tween and 5% non-fat milk. Membranes were then incubated overnight at 4°C with primary antibodies directed against the protein of interest. 4-HNE (Abcam, Cambridge, MA) was probed as a measurement indicative of oxidative stress. Proteolytic activity was assessed by analyzing cleaved (active) calpain-1

(Cell Signaling) and cleaved (active) caspase-3 (Cell Signaling). Furthermore, α -II spectrin (Santa Cruz Biotechnology, Santa Cruz, CA) calpain-specific cleavage (145-kDa cleavage product) and caspase-3-specific cleavage (120-kDa cleavage product) were measured to obtain an additional measurement of both calpain-1 and caspase-3 activity during MV. Lastly, measurements were made of Atrogin-1 and MuRF-1 (ECM Biosciences, Versailles, KY) as markers of proteasome activity. Bax and Bcl-2 (Abcam) were measured as markers of NF- κ B transcription activity. I κ B- α (Santa Cruz Biotechnology) protein levels were measured to assess NF- κ B activity. Following incubation with primary antibodies, membranes were washed extensively with PBS-Tween and then incubated with secondary antibodies (GE Healthcare, Piscataway, NJ). After washing, a chemiluminescent system was used to detect labeled proteins (GE Healthcare). Membranes were developed using autoradiography film and images of the film were captured and analyzed using the 440CF Kodak Imaging System (Kodak, New Haven, CT). In addition, membranes were stripped and reprobed for α -tubulin (Santa Cruz Biotechnology) which served as a loading control. α -tubulin protein concentration was also quantified using the 440CF Kodak Imaging System (Kodak) and used to normalize proteins by dividing them by the corresponding tubulin concentration.

RNA Isolation and cDNA Synthesis—Total RNA was isolated from muscle tissue with TRIzol Reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Total RNA and RNA content (μ g/mg muscle) were evaluated by spectrophotometry. Total RNA (5 μ g) was then reverse transcribed with the Superscript III First-Strand Synthesis System for RT-PCR (Life Technologies), using oligo(dT)20 primers and the protocol outlined by the manufacturer.

Real-time Polymerase Chain Reaction—One microliter of cDNA was added to a 25 μ l PCR reaction for real-time PCR using Taqman chemistry and the ABI Prism 7000 Sequence Detection system (Applied Biosystems, Foster City, CA). Relative quantification of gene expression was performed using the comparative computed tomography method (ABI, User Bulletin no. 2). This method uses a single sample, the calibrator sample (- *glucuronidase*; GenBank NM Y00717, NM M13962), for comparison of every unknown sample's gene expression. $CT[CT(\text{calibrator}) - CT(\text{sample})]$ was then calculated for each sample and relative quantification was calculated as 2^{-CT} . -*Glucuronidase*, a lysosomal glycoside hydrolase, was chosen as the reference gene based on previous work showing unchanged expression with our experimental manipulations (15, 16). Fivefold dilution curves were assayed on selected samples to confirm the validity of this quantification method for each gene. MAFbx, MuRF-1 and Nedd4 mRNA transcripts were assayed using predesigned rat primer and probe sequences commercially available from Applied Biosystems (Assays-on-Demand).

20S Proteasome Activity—A section of the ventral costal diaphragm was homogenized 1:10 (v:v) (5 mM Tris-HCL, pH = 7.5; 5 mM EDTA, pH = 8.0) and centrifuged at 1,500 $\times g$ for 10 minutes (4°C). Cytosolic fraction was centrifuged at 10,000 $\times g$ for 10 minutes (4°C) followed by an additional spin of the supernatant at 100,000 $\times g$ for one hour (4°C). The *in vitro* chymotrypsin-like activity of the 20S proteasome was measured fluorometrically using techniques described by Stein and co-workers (17).

p50 Activity—Cellular fractionation of diaphragm tissue was performed according to Alway et al (18). Diaphragmatic p50 DNA binding was measured in the nuclear protein fraction according to manufacturer's instructions using a commercially available NF- κ B Transcription Factor Kit (Thermo Scientific, Rockford, IL).

Measurement of *In Vitro* Diaphragmatic Contractile Properties—Upon sacrifice or the completion of the mechanical ventilation period, the entire diaphragm was removed and placed in a dissecting chamber containing a Krebs-Hensleit solution equilibrated with 95% O₂-5% CO₂ gas. A muscle strip, including the tendinous attachments at the central tendon and rib cage was dissected from the midcostal region. The strip was suspended vertically between two lightweight Plexiglas clamps with one end connected to an isometric force transducer (model FT-03, Grass Instruments, Quincy, MA) within a jacketed tissue bath. The force output was recorded via a computerized data-acquisition system (Super Scope II, GW Instruments Somerville, MA; Apple Computer Cupertino, CA). The tissue bath was filled with Krebs-Hensleit saline and the buffer was aerated with gas (95% O₂-5% CO₂), pH was maintained at 7.4, and the osmolality of the bath was ~290 mosmol/kgH₂O. After a 15-min equilibration period (25°C), *in vitro* diaphragmatic contractile measurements were made. The muscle strip was stimulated along its entire length with platinum wire electrodes (modified S48 stimulator, Grass Instruments) by using supramaximal (~150%) stimulation voltage to determine the optimum contractile length (*L_o*). *L_o* was determined by systematically adjusting the length of the muscle using a micrometer while evoking single twitches. Thereafter, all contractile properties were measured isometrically at *L_o*. To measure maximal isometric twitch force each strip was stimulated supramaximally with 120-V pulses at 1 Hz and to measure the force frequency response each strip was stimulated supramaximally with 120-V pulses at 15–160 Hz. The duration of each train was 500 ms to achieve a force plateau. Contractions were separated by a 2-min recovery period. For comparative purposes, diaphragmatic (bundles of fibers) force production was normalized as specific Po. The total muscle cross-sectional area at right angles to the long axis was calculated by the following algorithm (19): Total muscle cross-sectional area (mm²) = [muscle mass/(fiber length × 1.056)], where 1.056 is the density of muscle (in g/cm³). Fiber length was expressed in centimeters measured at *L_o* (20).

Histological Measures

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 (9). CSA was determined using Scion software (NIH).

Statistical Analysis

Comparisons between groups for each dependent variable were made by a one-way analysis of variance (ANOVA) and, when appropriate, a Tukey HSD (honestly significant difference) test was performed *post-hoc*. Significance was established at $p < 0.05$. Data are presented as means ± SD.

RESULTS

Systemic and Biologic Response to MV

Prior to the initiation of MV, no significant differences existed in body weight between the groups (CON = 288 g ± 4, 12MV = 295 g ± 5, 12MV-SN50 = 304 g ± 5 and 12MVC = 286 g ± 5). Importantly, 12-hours of MV did not significantly alter body weight of the MV, MV-SN50 or MVC group ($p < 0.05$). Note that heart rate (HR) and systolic blood pressure (SBP) were maintained relatively constant during the 12 hours of MV and no significant differences existed between experimental groups in any of these measures at the completion of 12 hours of MV (Table 1). The arterial partial pressures of O₂ (PaO₂) and CO₂ (PaCO₂) were also maintained relatively constant during MV with no significant differences existing between groups (Table 1). In addition the colonic (body) temperature (T) was also

maintained (36°C–37°C) during the MV period. At the completion of the MV protocols, there were no visual abnormalities of the lungs or peritoneal cavity, no visible indication of lung injury, and no evidence of infection, indicating that our aseptic surgical technique was successful.

NF- κ B Activity

The NF- κ B family of transcription factors is activated during conditions of disuse locomotor skeletal muscle atrophy. We measured p50 DNA binding in the diaphragm as a marker of NF- κ B activation. Our results indicate that p50 activity is increased during MV and that both SN50 and curcumin treatment attenuates the increase in p50 activation (Figure 1A). Because I κ B- binds NF- κ B family members preventing dimerization and activation, we also measured diaphragmatic levels of I κ B- following MV. Prolonged MV resulted in a significant decrease in diaphragmatic levels of I κ B- indicating that I κ B- was being degraded and providing further support that NF- κ B is activated in the diaphragm during MV (Figure 1B). Treatment with SN50 or curcumin decreased degradation of I κ B- in the diaphragm. In addition, the ratio of the pro-apoptotic protein Bax to the anti-apoptotic protein Bcl-2 was assessed to evaluate abundance of these two NF- κ B gene targets. Although not statistically significant, diaphragmatic levels of Bax tended to increase during MV whereas Bcl-2 levels tended to decrease. However, the ratio of Bax to Bcl-2 in the diaphragm was significantly increased during MV compared to both control and animals treated with the antioxidant curcumin and the NF- κ B inhibitor, SN50 (Figure 2 A–C).

Ubiquitin-Proteasome System

The ubiquitin-proteasome system of proteolysis is activated in the diaphragm during prolonged MV and therefore, is predicted to contribute to MV-induced diaphragmatic protein breakdown. To determine the effect of NF- κ B inhibition on the activity of the ubiquitin-proteasome system in the diaphragm, we measured 20S proteasome activity along with both mRNA and protein levels of the muscle specific E3 ligases (i.e., atrogin-1/MAFbx and MuRF-1) in the diaphragm. Our results reveal that both SN50 and curcumin administration during MV prevented the increase in 20S proteasome activity in the diaphragm (Figure 3A). We also observed a significant MV-induced increase in mRNA levels of the E3 ligase Nedd4 in the diaphragm and curcumin administration was sufficient to attenuate this expression (Figure 3B). Additionally, our data indicate that prolonged MV resulted in a significant increase in both diaphragm atrogin-1/MaFbx and MuRF-1 mRNA and protein levels (Figure 3C–F). Treatment with SN50 significantly blunted the increase in MuRF-1 mRNA and protein levels in the diaphragm but did not attenuate MV-induced increases in atrogin-1 mRNA and protein levels. Finally, curcumin administration attenuated increases in 20S proteasome activity along with the increases in mRNA and proteins levels of both atrogin-1 and MuRF-1 in the diaphragm.

Calpain and Caspase-3 Activity

Prior work by our group indicates that calpain and caspase-3 activation in the diaphragm plays an important role in MV-induced diaphragm weakness (7, 9). Therefore, we determined whether SN50 or curcumin treatment affected either calpain or caspase-3 activity in the diaphragm during MV. Treatment with curcumin significantly attenuated MV-induced increases in calpain activation; however, calpain activity remained significantly higher than control levels with SN50 treatment during MV (Figure 4A). In addition, both SN50 and curcumin treatment significantly attenuated increases in diaphragmatic caspase-3 activity during MV (Figure 4B). To confirm these results, we also measured calpain and caspase-3 specific cleavage of α -II spectrin (Figure 4C–D). These results were in general agreement with our direct measures of calpain and caspase-3 activity

and provide confirmatory evidence regarding the impact of SN50 and curcumin on MV-induced activation of calpain and caspase-3 in the diaphragm.

Oxidative Stress

The efficacy of curcumin to prevent MV-induced diaphragmatic oxidative stress was determined by measuring a biomarker of lipid peroxidation. 4-hydroxynoneal (4-HNE) is formed during the lipid peroxidation cascade and 4-HNE/protein adducts are an excellent biomarker of lipid peroxidation in tissue. Compared to control, 12 hours of MV resulted in a significant increase in diaphragmatic 4-HNE/protein adducts (Figure 5). Curcumin administration significantly attenuated the MV-induced accumulation of 4-HNE/protein adducts in the diaphragm. We interpret these findings as evidence that curcumin administration effectively inhibits MV-induced diaphragmatic oxidative stress. In contrast, treatment of animals with SN50 did not significantly prevent MV-induced oxidative stress in the diaphragm as evidenced by the significant increase in 4-HNE/protein adducts (Figure 5).

MV-induced Diaphragmatic Contractile Dysfunction

To evaluate diaphragmatic contractile dysfunction, we measured both the *in vitro* maximal isometric twitch force and force-frequency responses in strips of diaphragm muscle. Compared to control, 12-hours of MV significantly shifted the force-frequency response down and to the right at all stimulation frequencies (Figure 6). Prevention of NF- κ B activation via SN50 treatment protected the diaphragm against MV-induced contractile dysfunction at stimulation frequencies > 60Hz. Further, treatment with the antioxidant curcumin during MV significantly attenuated the MV-induced diaphragmatic contractile dysfunction at stimulation frequencies > 30Hz.

MV-induced Diaphragm Myofiber Atrophy

Myofiber cross-sectional area was determined for individual fiber types in diaphragm muscle strips from all treatment groups. Prolonged MV resulted in significant atrophy of type I, type IIa, and type IIb/IIx diaphragm myofibers. Importantly, treatment of animals with both SN50 and curcumin significantly attenuated MV-induced atrophy of type I and type IIa diaphragm myofibers (Figure 7). Finally, curcumin attenuated the MV-induced decrease in type IIb/x diaphragm fibers but SN50 failed to protect these fibers against atrophy.

DISCUSSION

Overview of Principle Findings

These experiments provide new and important findings relative to the signaling pathways responsible for the rapid onset of MV-induced diaphragm atrophy and contractile dysfunction. Specifically, our results are the first to establish the importance of NF- κ B activation in MV-induced diaphragm weakness and support the hypothesis that NF- κ B signaling contributes to MV-induced diaphragmatic atrophy and contractile dysfunction. Further, our findings corroborate our prediction that MV-induced oxidative stress is required for MV-induced NF- κ B activation in the diaphragm. Importantly, our discovery that the antioxidant curcumin is protective against both MV-induced oxidative stress and diaphragmatic weakness may have clinical implications regarding the use of curcumin or similar compounds to protect the diaphragm during prolonged MV. A detailed discussion of these important findings follows.

NF- κ B activation contributes to MV-induced diaphragm atrophy and contractile dysfunction

NF- κ B represents a family of five transcription factors, and active NF- κ B dimers can be formed by various combinations of these family members. It is well known that muscle disuse leads to increased transcriptional activity of NF- κ B (10, 11). Extensive work by Kandarian and colleagues has shown that knockout of either p50 or the co-transactivator Bcl-3 is sufficient to rescue skeletal muscle from disuse-induced atrophy (10, 21, 22). Specifically, the transcription factors p50 and Bcl-3 appear to be essential for atrophy during periods of skeletal muscle disuse (21). In this regard, the role that NF- κ B plays in MV-induced diaphragmatic atrophy is unknown and thus formed the rationale for the current experiments. To determine the importance of NF- κ B in MV-induced diaphragmatic weakness, we utilized the synthetic peptide SN50 to inhibit nuclear translocation of the NF- κ B family member p50. We hypothesized that NF- κ B activation and signaling contributes to MV-induced diaphragm atrophy and contractile dysfunction, and our results support this postulate.

The current investigation, along with many previous studies has shown that prolonged MV results in a rapid development of diaphragmatic atrophy (5, 7, 23–26). Specifically, numerous studies indicate that 12 hours of MV is sufficient to result in increased biomarkers of oxidative damage, decreased contractile function and decreased muscle fiber cross-sectional area in the rat diaphragm (5, 7–9, 25). This rapid MV-induced diaphragm atrophy is due to a decrease in protein synthesis and the rapid increase in proteolysis that occurs in the diaphragm during MV (5, 27). Indeed, by comparison to the time course of disuse-induced proteolysis in limb skeletal muscle, MV-induced proteolysis in the diaphragm occurs at a much earlier time point (28).

Importantly, our results show that inhibition of NF- κ B activation via SN50 administration was sufficient to attenuate the MV-induced decrease in diaphragm muscle fiber cross-sectional area of both Type I and Type IIa fibers. In contrast, inhibition of NF- κ B did not protect against MV-induced atrophy of diaphragm Type IIb/x fibers. Future studies are necessary to examine why the Type I and Type IIa fibers were selectively protected. In addition, inhibiting NF- κ B activation resulted in only a partial protection of MV-induced diaphragmatic contractile dysfunction. Hence, while our results indicate that NF- κ B signaling contributes to MV-induced weakness in the diaphragm, our findings reveal that NF- κ B activation alone is not solely responsible for the signaling pathways leading to diaphragmatic weakness during prolonged MV. In this regard, our current and previous work suggests that activation of proteases (e.g. calpain and caspase-3) are also involved in MV-induced diaphragmatic atrophy and contractile dysfunction (7, 9).

It appears likely that NF- κ B activity contributes to muscle atrophy due to its ability to transcribe specific atrophy-related genes. When activated, NF- κ B rapidly enters the nucleus and promotes gene transcription. Specifically, NF- κ B activity is responsible for the transcription of many pro- and anti-apoptotic factors (i.e. Bax and Bcl-2) and the E3 ligase Nedd4 and MuRF-1 (10, 11, 29, 30). Bax is a pro-apoptotic protein that can promote mitochondrial outer membrane pore opening leading to the release of cytochrome c and activation of caspase-3. Bcl-2 is anti-apoptotic protein and increases in the Bax-to-Bcl-2 ratio are often used as a biomarker of the vulnerability of mitochondria to trigger apoptosis (31). Furthermore, the proteasome system plays an important role in muscle wasting due to its ability to degrade polyubiquitinated proteins; this augmented degradation of polyubiquitinated proteins is due, in part, to the increased expression of two muscle-specific ubiquitin ligases atrogin-1 and MuRF-1 (14). Our data reveal that during MV, that there is a significant increase in both the Bax-to-Bcl-2 ratio and caspase-3 activation in the diaphragm. Importantly, our findings reveal that treatment of animals with either an NF- κ B inhibitor

(i.e., SN50) or antioxidant (i.e., curcumin) was sufficient to inhibit both the MV-induced increase in the Bax-to-Bcl-2 ratio and caspase-3 activation in the diaphragm. Moreover, MV promoted an increased expression of Nedd4, Atrogin-1 and MuRF-1 in the diaphragm. This effect was inhibited by the antioxidant curcumin, whereas inhibition of NF- κ B activation attenuated MuRF-1 and Nedd4 expression but did not protect against Atrogin-1 expression in the diaphragm.

Oxidative stress is required for NF- κ B activation in the diaphragm during prolonged MV

It is clear that MV-induced oxidative stress is required for MV-induced diaphragmatic weakness (4, 5, 32). More specifically, our prior work reveals that MV-induced oxidative stress is a requirement for both the diaphragmatic atrophy and contractile dysfunction that is associated with prolonged MV (6, 8). Further, we have also shown that MV-induced oxidative stress is essential for the activation of numerous proteases in the diaphragm including calpain, and caspase-3 (6, 7). Activation of calpain and caspase-3 in the diaphragm is significant because both proteases are important contributors to MV-induced diaphragmatic proteolysis and weakness (9, 33).

Although NF- κ B signaling has been shown to be sufficient and required for normal muscle wasting during disuse-induced skeletal muscle atrophy, prior to the current experiments it was unknown if MV-induced diaphragmatic oxidative stress acts as an upstream regulator of NF- κ B signaling during MV (10, 11). Our results support the hypothesis that MV-induced oxidative stress in the diaphragm is required for NF- κ B activation and reveal that treatment of animals with the potent polyphenolic antioxidant curcumin protects against oxidative damage and prevents NF- κ B activation in the diaphragm during MV. Although the current experiments do not reveal the mechanism behind this protection, it is possible that curcumin prevents NF- κ B activation by inhibiting I κ B- phosphorylation and degradation by the proteasome (6, 34). It is also possible that curcumin may inhibit MV-induced diaphragmatic NF- κ B activation by preventing calpain and caspase-3 activation (7). Indeed, both active calpain and caspase-3 have also been shown to cleave I κ B- to activate NF- κ B (35, 36). Therefore, it is possible that the proteasome, calpain, and caspase-3 may all play an important role in NF- κ B activation and that curcumin prevents MV-induced diaphragmatic NF- κ B activation by preventing proteasome, calpain, and caspase-3 activation.

Curcumin protects against MV-induced diaphragmatic weakness

Notably, in addition to attenuating oxidative damage and NF- κ B activation in the diaphragm during MV, treatment of animals with the antioxidant curcumin protected against the MV-induced decreases in diaphragm muscle fiber cross-sectional area and contractile dysfunction. Further, curcumin protected against MV-induced diaphragm contractile dysfunction at frequencies greater than 30Hz. It is unclear why curcumin failed to protect the diaphragm at lower stimulation frequencies. Finally, these results are consistent with previous results indicating that the antioxidant trolox is also capable of preventing MV-induced diaphragmatic weakness (4, 6–8). The finding that curcumin protects against MV-induced diaphragmatic weakness has clinical implications and suggests that this compound could have therapeutic potential in the prevention of MV-induced diaphragmatic weakness.

In regard to the possible clinical application of curcumin in patients undergoing MV, a recent review has concluded that curcumin is safe to humans even in high doses (~2–12 grams) (37). Moreover, curcumin administration is associated with few unpleasant side effects in patients (38). Finally, curcumin administration to human appears safe over an extended period of time and therefore, extended administration of curcumin to patients during extended periods of MV is feasible.

Conclusions

This study provides the first evidence regarding the importance of NF- κ B signaling in MV-induced protease expression and weakness in the diaphragm. Specifically, our results reveal that NF- κ B activation is required for MV-induced expression of MuRF-1 in the diaphragm. Further, our findings indicate that NF- κ B signaling plays an important role in MV-induced diaphragmatic weakness as prevention of NF- κ B activation provided partial protection against both MV-mediated diaphragmatic atrophy and contractile dysfunction. Importantly, our results also demonstrate that oxidative stress is essential to activate NF- κ B in the diaphragm during MV and that the antioxidant curcumin protects the diaphragm against MV-induced weakness. Collectively, these results suggest that both the NF- κ B signaling pathway and/or oxidant production pathways are potential therapeutic targets to rescue the diaphragm from MV-induced atrophy and contractile dysfunction. Given that it appears likely that MV-induced diaphragmatic weakness is a contributor to problematic weaning, developing methods to prevent MV-induced diaphragmatic atrophy and contractile dysfunction remains an important clinical goal.

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Figure 1.

NF- κ B activation was assessed via (A) p50 activity and (B) I κ B- α degradation. A representative blot for I κ B- α protein is shown above the graph. Values are mean percentage change $\pm$ SD. $\S$ Significantly different versus all groups ($p < 0.05$). Control = acutely anesthetized, no mechanical ventilation (MV), 12MV = 12 hours of controlled MV, 12MVC = 12 hours of MV-treated with Curcumin, 12MV-SN50 = 12 hours of MV treated with SN50.

Figure 2.

(A) Bax and (B) Bcl-2 were assessed via Western blotting. (C) The ratio of Bax to Bcl-2 was also calculated. Representative blots for Bax and Bcl-2 protein are shown above the graph. Values are mean percentage change $\pm$ SD. [§] Significantly different versus all groups ($p < 0.05$). Control = acutely anesthetized, no mechanical ventilation (MV), 12MV = 12 hours of controlled MV, 12MVC = 12 hours of MV-treated with Curcumin, 12MV-SN50 = 12 hours of MV treated with SN50.

Figure 3.

(A) Chymotrypsin-like activity of the 20S ubiquitin-proteasome system. (B) mRNA levels of Nedd4. (C) mRNA levels of atrogin-1. (D) mRNA levels of MuRF-1. (E) Protein levels of atrogin-1. (F) Protein levels of MuRF-1. Representative blots for atrogin-1 and MuRF-1 protein is shown above the graph. Values are mean percentage change $\pm$ SD. $\S$ significantly different versus all groups ($p < 0.05$). $*$ Significantly different versus Control ($p < 0.05$). $\dagger$ Significantly different versus control and 12MVC ($p < 0.05$). Control = acutely anesthetized, no mechanical ventilation (MV), 12MV = 12 hours of controlled MV, 12MVC = 12 hours of MV-treated with Curcumin, 12MV-SN50 = 12 hours of MV treated with SN50.

Figure 4.

(A and C) Calpain (B and D) caspase-3 activation was determined via Western blotting for active calpain, cleaved caspase-3 and the spectrin break-down product (SBDP). Representative Western blots are shown above the graphs. Values are mean percentage change $\pm$ SD. * Significantly different versus Control. § Significantly different versus all groups ($p < 0.05$). † Significantly different versus control and 12MVC ($p < 0.05$). Control = acutely anesthetized, no mechanical ventilation (MV), 12MV = 12 hours of controlled MV, 12MVC = 12 hours of MV-treated with Curcumin, 12MV-SN50 = 12 hours of MV treated with SN50.

Figure 5.

The levels of 4 hydroxynonenal (4-HNE) were analyzed as an indicator of lipid peroxidation via Western blotting. A representative blot for 4-HNE protein conjugates is shown above the graph. Values are mean percentage change $\pm$ SD. † Significantly different versus Control and 12MVC ($p < 0.05$). ‡ Significantly different versus 12MVC ($p < 0.05$). Control = acutely anesthetized, no mechanical ventilation (MV), 12MV = 12 hours of controlled MV, 12MVC = 12 hours of MV-treated with Curcumin, 12MV-SN50 = 12 hours of MV treated with SN50.

Figure 6.

Diaphragmatic force-frequency response (in vitro). Values are mean $\pm$ SD. † Control significantly different versus 12MV and 12MVC ($p < 0.05$). § 12MV significantly different versus all groups ($p < 0.05$). * 12MV-SN50 significantly different versus Control. # 12MV-SN50 significantly different versus Control and 12MVC ($p < 0.05$). ‡ 12MVC significantly different versus Control and 12MV-SN50 ($p < 0.05$). ‡ 12MV significantly different versus 12MV-SN50 and Control. Control = acutely anesthetized, no mechanical ventilation (MV), 12MV = 12 hours of controlled MV, 12MVC = 12 hours of MV-treated with Curcumin, 12MV-SN50 = 12 hours of MV treated with SN50.

Figure 7.

Fiber cross-sectional area (CSA) in diaphragm skeletal muscle myofibers expressing myosin heavy chain (MHC) I (Type I), MHC IIa (Type IIa), and MHC IIb/IIx (Type IIb/IIx). **(A)** Representative fluorescent staining of MHC I (DAPI filter / blue), MHC IIa (FITC filter / green), and dystrophin (Rhodamine filter / red) proteins in diaphragm samples. **(B)** Type I, Type IIa, and Type IIb/IIx fiber cross-sectional area. Values are mean $\pm$ SD. § Significantly different versus all groups ($p < 0.05$). † Significantly different versus Control and 12MVC ($p < 0.05$). Control = acutely anesthetized, no mechanical ventilation (MV), 12MV = 12 hours

of controlled MV, 12MVC = 12 hours of MV-treated with Curcumin, 12MV-SN50 = 12 hours of MV treated with SN50.

Table 1

Animal heart rates, systolic blood pressure, arterial blood gas tensions, and arterial pH at the completion of 12 hours of mechanical ventilation.

Physiological variable	MV	MVC	MVSN50
Heart rate (beats/min)	369±23	354±14	363±23
Systolic blood pressure (mm/Hg)	97±23	104±23	111±23
Arterial PO ₂ (mm/Hg)	79±26	74±20	73±17
Arterial PCO ₂	36±11	34±9	35±14
Arterial pH	7.40±0.03	7.42±0.03	7.42±0.03

Values are means ± SD. Note that no significant differences existed between the two experimental groups in any of these physiological variables. MV = 12 hours of controlled MV, MVC = 12 hours of MV-treated with Curcumin, MVSN50 = 12 hours of MV treated with SN50.