ANESTHESIOLOGY

Oxidants Regulated Diaphragm Proteolysis during Mechanical Ventilation in Rats

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EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Diaphragm dysfunction and atrophy develop during controlled mechanical ventilation. Although oxidative stress injures muscle during controlled mechanical ventilation, it is unclear whether it causes autophagy or fiber atrophy.

What This Article Tells Us That Is New

- Pretreatment of rats undergoing 24 h of mechanical ventilation with *N*-acetylcysteine prevents decreases in diaphragm contractility, inhibits the autophagy and proteasome pathways, but has no influence on the development of diaphragm fiber atrophy.

ABSTRACT

Background: Diaphragm dysfunction and atrophy develop during prolonged controlled mechanical ventilation. Fiber atrophy has been attributed to activation of the proteasome and autophagy proteolytic pathways. Oxidative stress activates the proteasome during controlled mechanical ventilation, but it is unclear whether it also activates autophagy. This study investigated whether pretreatment with the antioxidant *N*-acetylcysteine affects controlled mechanical ventilation–induced diaphragm contractile dysfunction, fiber atrophy, and proteasomal and autophagic pathway activation. The study also explored whether proteolytic pathway activity during controlled mechanical ventilation is mediated by microRNAs that negatively regulate ubiquitin E3 ligases and autophagy-related genes.

Methods: Three groups of adult male rats were studied (n = 10 per group). The animals in the first group were anesthetized and allowed to spontaneously breathe. Animals in the second group were pretreated with saline before undergoing controlled mechanical ventilation for 24 h. The animals in the third group were pretreated with *N*-acetylcysteine (150 mg/kg) before undergoing controlled mechanical ventilation for 24 h. Diaphragm contractility and activation of the proteasome and autophagy pathways were measured. Expressions of microRNAs that negatively regulate ubiquitin E3 ligases and autophagy-related genes were measured with quantitative polymerase chain reaction.

Results: Controlled mechanical ventilation decreased diaphragm twitch force from 428 ± 104 g/cm^2 (mean ± SD) to 313 ± 50 g/cm^2 and tetanic force from 2,491 ± 411 g/cm^2 to 1,618 ± 177 g/cm^2. Controlled mechanical ventilation also decreased diaphragm fiber size, increased expression of several autophagy genes, and augmented *Atrogin-1*, *MuRF1*, and *Nedd4* expressions by 36-, 41-, and 8-fold, respectively. Controlled mechanical ventilation decreased the expressions of six microRNAs (miR-20a, miR-106b, miR-376, miR-101a, miR-204, and miR-93) that regulate autophagy genes. Pretreatment with *N*-acetylcysteine prevented diaphragm contractile dysfunction, attenuated protein ubiquitination, and downregulated E3 ligase and autophagy gene expression. It also reversed controlled mechanical ventilation–induced microRNA expression decreases. *N*-Acetylcysteine pretreatment had no affect on fiber atrophy.

Conclusions: Prolonged controlled mechanical ventilation activates the proteasome and autophagy pathways in the diaphragm through oxidative stress. Pathway activation is accomplished, in part, through inhibition of microRNAs that negatively regulate autophagy-related genes.

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Mechanical ventilation is a common medical procedure used for the maintenance of adequate gas exchange. Despite being indispensable, controlled mechanical ventilation results in complications that give rise to a pathology known as ventilator-induced diaphragm dysfunction[1] that is characterized by contractile dysfunction of the diaphragm, diaphragm fiber atrophy, and loss of myofilament proteins.[2–4] Ventilator-induced diaphragm dysfunction has been successfully reproduced in experimental animals.[5–7]

Controlled mechanical ventilation–induced diaphragm atrophy has been attributed to both decreased protein synthesis and increased protein degradation by the proteasomal, calpain, caspase-3, and autophagic proteolytic pathways.[8] Activation of these pathways during prolonged controlled

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mechanical ventilation has been attributed to increased production of reactive oxygen species.[4,7,9,10] Administration of antioxidants protects against impaired diaphragm contractility[7,11–13] and attenuates the activities of the proteasome, calpain, and caspase-3.[5,7,11,13]

It remains unclear, however, whether controlled mechanical ventilation–induced oxidative stress *per se* activates autophagy in diaphragm muscle fibers or what effect administration of antioxidants has on the degree of controlled mechanical ventilation–induced autophagy produced. It is also unknown whether antioxidants alter the degree of fiber atrophy that develops during controlled mechanical ventilation because most published reports do not include measurements of diaphragm fiber size. The first objective of the current study, therefore, is to determine how the antioxidant *N*-acetylcysteine affects contractile function, fiber atrophy, and proteasomal and autophagic pathway activation in diaphragm muscle fibers of rats undergoing 24 h of controlled mechanical ventilation. We hypothesize that controlled mechanical ventilation–induced increases in reactive oxygen species levels in the diaphragm simultaneously activate the proteasome and autophagy pathways and that administration of *N*-acetylcysteine before controlled mechanical ventilation attenuates pathway activity, thereby improving diaphragm contractile function and ameliorating fiber atrophy.

MicroRNAs are important epigenetic regulators of myogenesis and atrophy through their effects on the proteasome proteolytic pathway.[14] They are short noncoding RNA molecules that function primarily as inhibitors of gene and protein expression. The importance of microRNAs to the regulation of autophagy in skeletal muscles is unknown. Recent studies have identified several microRNAs in nonmuscle cells that target autophagy-related genes. For instance, miR-17, miR-20a, miR-30a, miR-101a, miR-106, miR-145, miR-204, miR-210a, and miR-376b have been shown to inhibit autophagy by selectively targeting ULK1 (Unc-51 Like Autophagy Activating Kinase 1), BECN1 (Beclin1), ATG4 (Autophagy Related 4a), LC3B (Microtubule Associated Protein 1 Light Chain 3 Beta), BNIP3 (BCL2 Interacting Protein 3), and SQSTM1 (Sequestosome 1).[15-24] Furthermore, the expressions of muscle-specific ubiquitin E3 ligases, Fbxo32 (F-Box Protein 32, Atrogin-1), and Trim63 (Tripartite Motif Containing 63, MuRF1) are downregulated by miR-23a.[25] Recently, two microRNAs, miR-21 and miR-206, have been shown to promote denervation-induced muscle atrophy.[26] The second objective of this study, therefore, is to determine whether *N*-acetylcysteine administration affects the expressions of specific microRNAs that regulate ubiquitin E3 ligases or autophagy-related genes in the diaphragms of rats undergoing 24 h of controlled mechanical ventilation. We hypothesize that increased expressions of ubiquitin E3 ligases and autophagy-related genes in the diaphragms during prolonged controlled mechanical ventilation is a result, in part, of selective downregulation of their corresponding microRNAs and that this response is mediated through the production of reactive oxygen species within diaphragm muscle fibers.

Materials and Methods

Experimental Procedure and Study Design

Research protocols were approved by the Animal Experiments Committee of the Medical Faculty of Katholieke Universiteit, Leuven, Belgium. Adult male Wistar rats (400 to 500 g, 12 to 16 weeks old) were randomly assigned to three groups of 10 animals: 1) spontaneously breathing anesthetized animals; 2) mechanically ventilated (24 h) animals pretreated with equal volumes of normal saline; and 3) mechanically ventilated (24 h) animals pretreated with 150 mg/kg *N*-acetylcysteine. The three groups of animals were examined in random order. An intravenous bolus of either normal saline or *N*-acetylcysteine was injected at the beginning of the study period, before starting mechanical ventilation. *N*-Acetylcysteine dosages that have been previously shown to be effective in preventing diaphragm contractile dysfunction in rats[11] and mice[5] were used. Controlled mechanical ventilation was performed as previously described.[27] Before being tracheostomized, animals were anesthetized with sodium pentobarbital (60 mg/kg). The right external jugular vein and carotid artery were catheterized to allow for continuous infusion of sodium pentobarbital and heparin, respectively. Animals breathed O_2-enriched humidified air maintained at 37°C. Controlled mechanical ventilation was applied with a volume-driven small-animal ventilator (tidal volume ± 0.5 ml/100g; breathing frequency, 55 to 60 breaths/min). Body temperature was continuously measured and maintained at 37°C using a heated blanket. Arterial blood pressure was monitored, and blood gases were measured initially and after 12 and 24 h of controlled mechanical ventilation. No nutritional support was provided during the experimental period. After 24 h, the animals were euthanized with an overdose of pentobarbital, and segments of the costal diaphragm were removed to assess diaphragm contractility (see Diaphragm Contractility section). A separate piece of the right diaphragm was used to determine cross-sectional area, minimum Feret diameter of muscle fibers, and fiber type distribution (see Muscle Fiber Atrophy section). The remainder of the diaphragm and the gastrocnemius muscle (representative of limb muscle, analyzed for comparative purposes) were frozen in liquid nitrogen and stored at −80°C.

Diaphragm Contractility

Diaphragm contractility was measured as previously described.[28] In brief, diaphragm muscle bundle contractility was measured *in vitro* at 37°C using a temperature-controlled organ bath and stimulating electrodes. Optimal muscle length (L_o) for peak twitch force was established. Maximal tetanic force was measured at L_o with a stimulation

frequency of 160 Hz. The following measurements were performed at L_o: 1) twitch characteristics (maximal twitch tension was obtained from one twitch stimulation [1 Hz]) and 2) maximal tetanic force (bundles were stimulated twice at 160 Hz, during 250 ms, with a 2-min interval). Each pulse had a duration of 0.2 ms. Tetanic force was taken as the maximal tension elicited at 160 Hz. Muscle force was normalized for bundle cross-sectional areas (obtained by dividing bundle weight by muscle specific density and optimal length).

Muscle Fiber Atrophy

Diaphragm muscle samples frozen in liquid nitrogen-cooled isopentane were cut into 10-μm cross-sections and stained using a selective antibody for LAMININ protein (Supplemental Digital Content, http://links.lww.com/ALN/C15). Images were captured using a microscope. Minimum Feret diameters of at least 600 fibers/muscle cross-section were measured by a single observer blinded to the identity of the samples. To assess whether diaphragm fiber atrophy is selective to a specific fiber type, diaphragm cross-sections were stained for myosin heavy chain type I, IIa, and IIb proteins (Supplemental Digital Content, http://links.lww.com/ALN/C15). Minimum Feret diameters of each fiber type were measured by a single observer blinded to the identity of the samples.

Immunoblotting

Frozen diaphragm tissue samples were prepared for immunoblotting as previously described.[29] Specific proteins were detected using an enhanced chemiluminescence kit and primary antibodies (Supplemental Digital Content, http://links.lww.com/ALN/C15). Immunoblots were scanned with an imaging densitometer, and optical densities of protein bands were quantified.

Protein Carbonylation

To detect the effects of mechanical ventilation and *N*-acetylcysteine administration on the development of oxidative stress, protein carbonylation was measured in the diaphragm as previously described[30] (Supplemental Digital Content, http://links.lww.com/ALN/C15).

mRNA Detection

Total RNA from diaphragm samples and cultured murine C2C12 cells was extracted and prepared for real-time polymerase chain reaction detection of various transcripts (Supplemental Digital Content, http://links.lww.com/ALN/C15). The primers were designed to quantify the expressions of specific mRNAs (Supplemental Digital Content, http://links.lww.com/ALN/C15). β-Actin served as an endogenous control transcript. Relative mRNA level quantifications of target genes were determined using the threshold cycle ($\Delta\Delta C_T$) method.

Ubiquitin–Proteasome Pathway Measurements

Proteolysis by the proteasome system was evaluated by measuring total protein ubiquitination with immunoblotting and by quantifying mRNA expressions of the ubiquitin E3 ligases *Fbxo32* (*Atrogin-1*), *Trim63* (*Murf-1*), *Nedd4* (neural precursor cell-expressed developmentally down-regulated protein 4), and *Fbxo30* (*Musa1*) with quantitative polymerase chain reaction (Supplemental Digital Content, http://links.lww.com/ALN/C15).

Autophagy Measurements

Autophagosomes develop *via* a cascade of tightly regulated steps from membrane commitment and elongation to final formation. The expressions of several autophagy-related genes were measured across this cascade using immunoblotting and quantitative real-time polymerase chain reaction, as previously described.[31,32]

Regulators of Protein Synthesis and Degradation

To evaluate the activities of protein kinase B (AKT), mammalian target of rapamycin complex 1, and adenosine monophosphate (AMP)-activated protein kinase α, phosphorylation levels of AKT on Ser^{473}, P70S6K1 (Ribosomal Protein S6 Kinase B1) on Thr^{389}, and AMP-activated protein kinase α on Thr^{172}, respectively, were measured using immunoblotting. P70S6K1 is a serine/threonine kinase that phosphorylates the ribosomal protein S6 and is downstream from mammalian target of rapamycin complex 1. Expressions of *Foxo1* and *Foxo3* transcription factors (targets of inhibitory effects of AKT) were also measured, using quantitative polymerase chain reaction.

MicroRNA Detection

Total RNA was extracted from frozen muscle samples and C2C12 cell lysates using Qiazol and a Qiagen (Germany) miRNeasy mini kit, according to the manufacturer's protocols. Mature microRNAs were detected using an NCode microRNA quantitative real-time polymerase chain reaction kit and real-time polymerase chain reaction with specific primers (Supplemental Digital Content, http://links.lww.com/ALN/C15), SYBR green, and a 7500 real-time polymerase chain reaction system. Relative microRNA expression was determined using the C_T method, where C_T values of individual microRNA data were normalized to C_T values of U6 snRNA, as previously described.[33]

Myoblast Exposure to Hydrogen Peroxide

To investigate the effects of reactive oxygen species on the regulation of autophagy-related genes and their corresponding microRNAs, murine C2C12 skeletal myoblasts were exposed to hydrogen peroxide. The cells were maintained in culture (Supplemental Digital Content, http://links.lww.com/ALN/C15) and exposed for 24 h to

phosphate-buffered saline (control condition) or hydrogen peroxide (100 μM). The cells were lysed, total RNA and proteins were extracted, and mRNA levels of selected autophagy-related genes (*Ulk1*, *Becn1*, *Atg4b*, *Bnip3*, *Lc3b*, and *Sqstm1*) were detected using quantitative polymerase chain reaction, as were the levels of microRNAs that regulate their expression (Supplemental Digital Content, http://links.lww.com/ALN/C15). Immunoblotting was used to detect lipidation of LC3B protein (LC3B-II protein intensity) as an index of the degree of autophagosome formation.

Statistical Analyses

Statistical analyses were performed using a SAS Statistical package and SigmaStat software. Normal distribution was tested with a D'Agostino and Pearson omnibus normality test. All data are expressed as means ± SD except for minimum Feret diameters of diaphragm muscle fibers, where data are presented using a box-and-whisker plot. Comparisons between the three experimental groups were performed using one-way ANOVA, followed by Tukey *post hoc* tests except for minimum Feret diameters. To compare minimum Feret diameters of diaphragm muscle fibers of the three groups, we performed a Kruskal–Wallis test for between-group comparisons, followed by Dunn's *post hoc* tests and Bonferroni correction for multiple comparison. The number of rats per group required to identify differences in major physiologic parameters was estimated based on previous experience with this model, and there is no missing information from any of the reported data sets. No formal statistical power calculation was conducted. All statistical tests are two-tailed. Significance was established at $P < 0.05$.

Results

Diaphragm Contractility

Diaphragm twitch force (313 ± 50 g/cm^2) and maximum tetanic force (1,618 ± 177 g/cm^2) were significantly lower in the mechanical ventilation groups as compared with the spontaneously breathing group (428 ± 104 and 2,491 ± 411 g/cm^2, respectively; fig. 1A). Administration of *N*-acetylcysteine in the mechanical ventilation-NAC group prevented controlled mechanical ventilation–induced diaphragm contractile dysfunction, as indicated by restoration of twitch (606 ± 69 g/cm^2) and maximum tetanic forces (2,167 ± 234 g/cm^2; fig. 1A).

Diaphragm Fiber Atrophy

Minimum Feret diameters of diaphragm muscle fibers were significantly lower in the mechanical ventilation group as compared with the spontaneously breathing group (fig. 1B). Decreases in the mechanical ventilation group were observed in all fiber types (Supplemental Digital Content, fig. 1, http://links.lww.com/ALN/C15). Minimum Feret diameters in the mechanical ventilation-NAC group were not significantly different from those of the mechanical ventilation group but were significantly lower than those of the spontaneously breathing group (fig. 1B). This was true for all fiber types (Supplemental Digital Content, fig. 1, http://links.lww.com/ALN/C15).

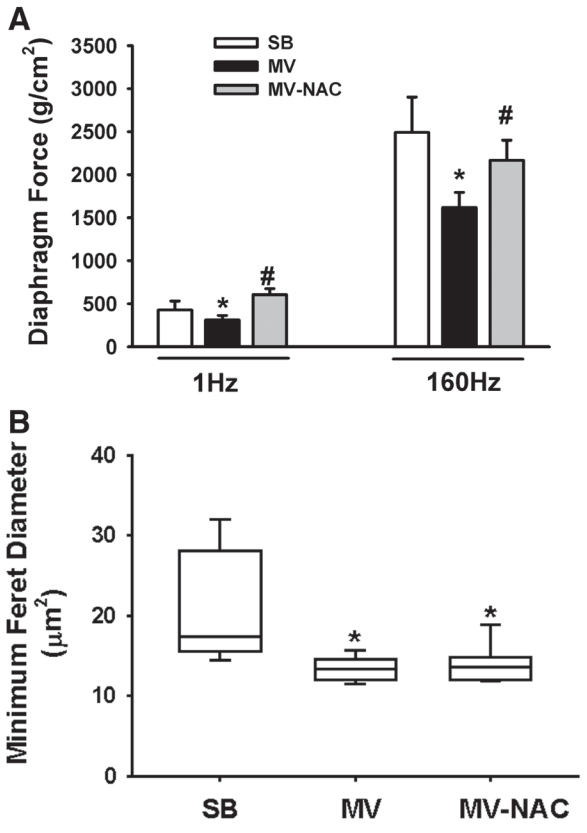

Fig. 1. (*A*) Peak diaphragm force generation in response to stimulation of 1 and 160 Hz in the group allowed to spontaneously breathe (SB; n = 10), the group pretreated with saline before undergoing controlled mechanical ventilation for 24 h (MV; n = 10), and the group pretreated with *N*-acetylcysteine before undergoing controlled mechanical ventilation for 24 h (MV-NAC; n = 10), where n refers to the number of animals. The values are means ± SD. *$P < 0.05$, compared with the SB group; #$P < 0.05$, compared with the MV group. Comparisons between the three experimental groups were performed using one-way ANOVA. (*B*) Minimum Feret diameters of diaphragm muscle fibers in the SB (n = 7), MV (n = 6), and MV-NAC (n = 6) groups. The data are presented using a box-and-whisker plot. *$P < 0.05$, compared with SB group. Comparisons between the three groups were performed using Kruskal–Wallis test followed by Dunn's *post hoc* tests and Bonferroni correction for multiple comparison.

Protein Carbonylation

To assess the effects of controlled mechanical ventilation and *N*-acetylcysteine administration on protein oxidation as an index of oxidative stress, total carbonyl content in the

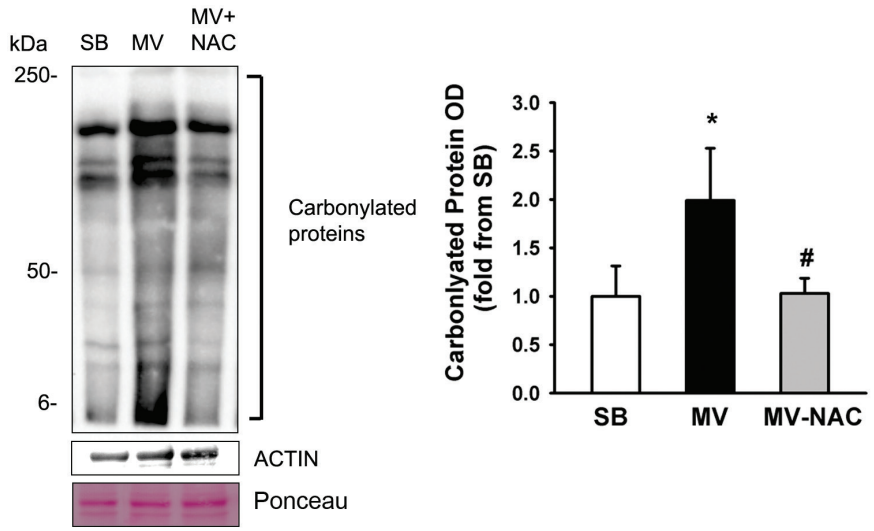

Fig. 2. Representative immunoblots and total optical density (OD) of protein carbonylation in the diaphragms by group; n = 5 per group, where n refers to the number of animals. The values are means ± SD. *$P < 0.05$, compared with the group allowed to spontaneously breathe (SB); #$P < 0.05$, compared with the group pretreated with saline before undergoing controlled mechanical ventilation for 24 h (MV). Comparisons between the three experimental groups were performed using one-way ANOVA. MV-NAC, group pretreated with *N*-acetylcysteine before undergoing controlled mechanical ventilation for 24 h.

diaphragm was measured using immunoblotting. Total protein carbonylation in the diaphragm of the mechanical ventilation group significantly increased (1.99 ± 0.53-fold) as compared with the spontaneously breathing group (fig. 2). *N*-Acetylcysteine administration completely reversed controlled mechanical ventilation–induced increases in diaphragm protein carbonylation (1.03 ± 0.15-fold; fig. 2).

Proteasome Pathway

Total protein ubiquitination (1.8 ± 0.3-fold) and *Fbxo32* (*Atrogin-1*; 36.1 ± 13.8-fold), *Trim63* (*MuRF1*; 41.3 ± 17.9-fold), and *Nedd4* (8.4 ± 1.3-fold) mRNA expressions significantly increased in the diaphragms of the mechanical ventilation group as compared with the spontaneously breathing group (fig. 3, A and B). *N*-Acetylcysteine administration completely reversed controlled mechanical ventilation–induced increases in protein ubiquitination, attenuated mRNA expression increases of *Fbxo32* (17.1 ± 6.7-fold) and *Trim63* (15.5 ± 7.8-fold), had no effect on *Nedd4* (8.6 ± 1.2-fold), and led to decreased *Fbxo30* (*Musa1*; 0.51 ± 0.09-fold) mRNA levels in comparison with the spontaneously breathing and mechanical ventilation groups (fig. 3C).

Autophagy Pathway

Ulk1 is an index of autophagy initiation; *Beclin1* (Becn1) and *Uvrag* (UV Radiation Resistance Associated) are indicators of the membrane nucleation process; *Lc3b*, *Gabarapl1* (γ-Aminobutyric Acid Type A Receptor Associated Protein Like 1) and *Atg4b* are indicators of autophagosome membrane elongation; and *Bnip3*, *Sqstm1* (p62), and *Park2* are markers of mitophagy. mRNA expressions of *Ulk1* (13.9 ± 3.5-fold), *Atg4b* (4.9 ± 1.1-fold), *Becn1* (5.9 ± 1.6-fold), *Gabarapl1* (11.2 ± 2.7-fold), *Sqstm1* (14.2 ± 3.8-fold), *Uvrag* (4.3 ± 1.3-fold), and the transcription factors *Foxo1* (3.6 ± 1.3-fold) and *Foxo3* (3.1 ± 0.6-fold) significantly increased in the diaphragms of the mechanical ventilation group as compared with the spontaneously breathing group (fig. 4A). *N*-Acetylcysteine administration attenuated the degree to which mechanical ventilation upregulated *Ulk1*, *Atg4b*, *Garabarapl1*, *Sqstm1*, and *Foxo1* expressions but had no effect on controlled mechanical ventilation–induced increases in *Becn1*, *Uvrag*, and *Foxo3* (fig. 4A).

Protein levels of BECN1, BNIP3, SQSTM1, and PARKIN were measured using immunoblotting, as was the lipidation of cytosolic LC3B protein (LCB3-I) to LC3B-II, which is incorporated into the autophagosome membrane. LC3B-II protein intensity was used as an indicator of increasing incorporation into the membrane. LC3B-II (2.5 ± 0.8-fold), SQSTM1 (5.0 ± 0.9-fold), BECN1 (2.1 ± 0.4-fold), BNIP3 (1.9 ± 0.3-fold), and PARKIN (3.6 ± 0.9-fold) protein levels in the diaphragm significantly increased in the mechanical ventilation group as compared with the spontaneously breathing group (fig. 4, B and C). *N*-Acetylcysteine administration attenuated the degree to which mechanical ventilation induced increases in LC3B-II (1.3 ± 0.2-fold), SQSTM1 (2.2 ± 0.9-fold), BNIP3 (0.9 ± 0.2-fold), and PARKIN (0.9 ± 0.4-fold) protein levels but had no effect on BECN1 levels (2.0 ± 0.6-fold; fig. 4, B and C).

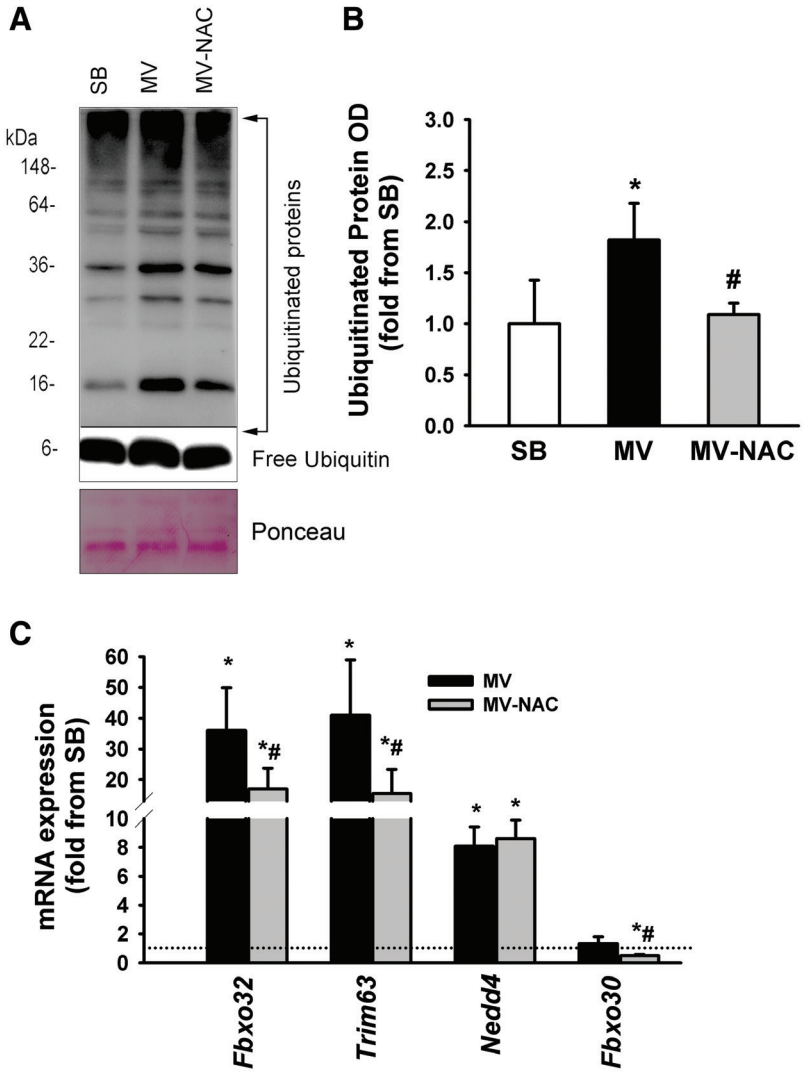

Fig. 3. (*A*, *B*) Representative immunoblots and optical densities (OD) of protein ubiquitination in diaphragms of the group allowed to spontaneously breathe (SB), the group pretreated with saline before undergoing controlled mechanical ventilation for 24 h (MV), and the group pretreated with *N*-acetylcysteine before undergoing controlled mechanical ventilation for 24 h (MV-NAC). The values are means ± SD, expressed as fold from SB values. **P* < 0.05, compared with SB group; #*P* < 0.05, compared with MV group. (*C*) mRNA expressions of *Fbxo32* (F-Box Protein 32), *Trim63* (Tripartite Motif Containing 63, *MuRF1*), *Nedd4* (Neural Precursor Cell Expressed, Developmentally Down-Regulated 4), and *Fbxo30* (F-Box Protein 30, *Musa1*) in diaphragms of MV and MV-NAC groups (n = 6 per group), where n refers to the number of animals. The values are means ± SD, expressed as fold change from SB group (*dotted line*). **P* < 0.05, compared with the SB group; #*P* < 0.05, compared with the MV group. Comparisons between the three experimental groups were performed using one-way ANOVA.

Regulators of Protein Synthesis and Degradation

Skeletal muscle protein synthesis is stimulated by several growth factors whose signaling pathways converge on the AKT and mammalian target of rapamycin complex 1 pathways. These pathways inhibit autophagy through inactivation of FOXO transcription factors and phosphorylation and inhibition of ULK1[34]. Another important regulator of autophagy is the AMP-activated protein kinase pathway, which activates autophagy through phosphorylation and activation of ULK1[35]. To evaluate mammalian target of rapamycin complex 1 and AMP-activated protein kinase α activities, the phosphorylation of AKT (Ser^{473}), P70S6K1 (Thr^{389}), and AMP-activated protein kinase α (Thr^{172}) were measured using immunoblotting. Relative to the spontaneously breathing group, phosphorylation levels of AKT (Ser^{473}; 0.8 ± 0.3-fold) and AMP-activated protein kinase α (Thr^{172}; 1.3 ± 0.4-fold) in the diaphragms of the mechanical ventilation group were similar, whereas P70S6K1 (Thr^{389}; 5.9 ± 2.4-fold) levels significantly increased (Supplemental Digital Content, fig. 2, http://links.lww.com/ALN/C15).

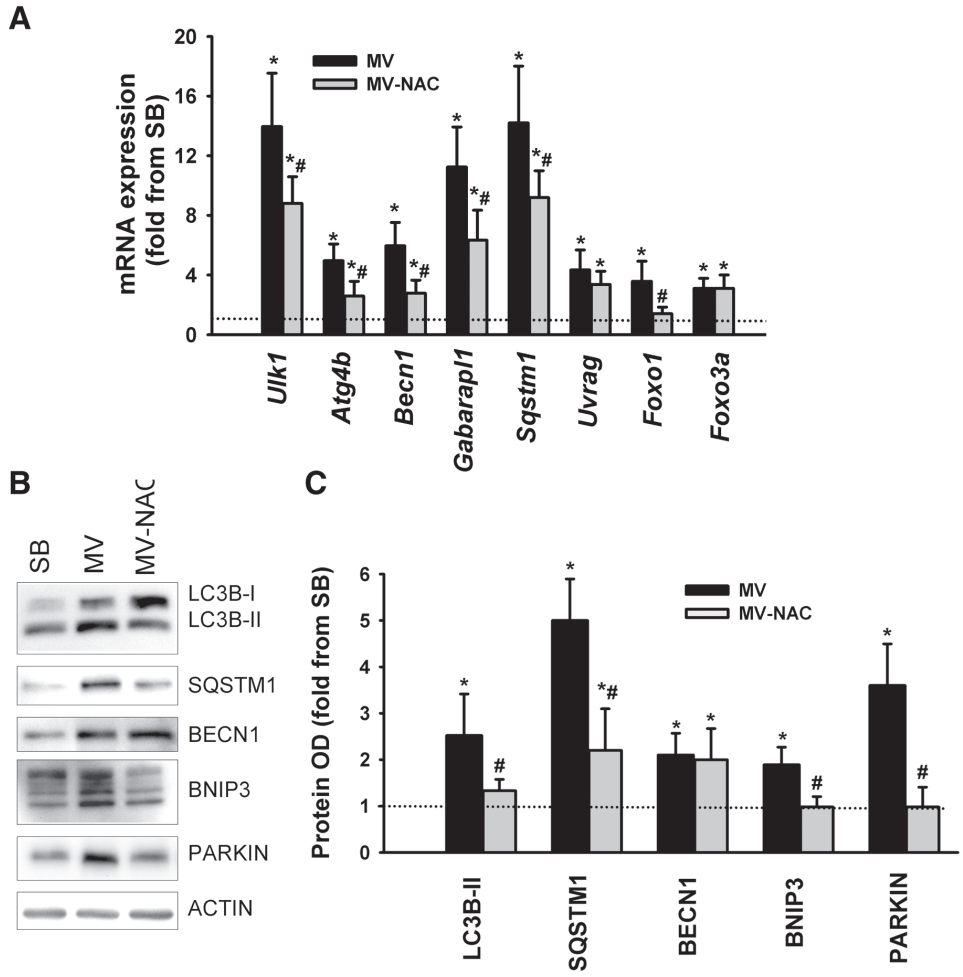

Fig. 4. (*A*) mRNA expressions of *Ulk1* (Unc-51 Like Autophagy Activating Kinase 1), *Becn1* (Beclin1), *Atg4b* (Autophagy Related 4a), *Bnip3* (BCL2 Interacting Protein 3), *Sqstm1* (Sequestosome 1), *Park2* (Parkin), *Uvrag* (UV Radation Resistance Associated 1), *Gabarapl1* (γ-Aminobutyric Acid Type A Receptor Associated Protein Like 1), *Foxo1* (Forhead Box 01), and *Foxo3* (Forkhead Box 03) in diaphragms of the groups pretreated with saline before undergoing controlled mechanical ventilation for 24 h (MV) and pretreated with *N*-acetylcysteine before undergoing controlled mechanical ventilation for 24 h (MV-NAC). (*B*, *C*) Representative immunoblots and optical densities (OD) of LC3B-II, SQSTM1 (p62), BECN1, PARKIN, and β-ACTN proteins in diaphragms of MV and MV-NAC groups (n = 6 per group), where n refers to the number of animals. The values are means ± SD, expressed as fold change from the group allowed to spontaneously breathe (SB; *dotted line*). **P* < 0.05, compared with the SB group; #*P* < 0.05, compared with the MV group. Comparisons between the three experimental groups were performed using one-way ANOVA.

N-Acetylcysteine administration had no effect on AKT or AMP-activated protein kinase α phosphorylation but eliminated controlled mechanical ventilation–induced increases in P70S6K1 phosphorylation (1.4 ± 0.8-fold; Supplemental Digital Content, fig 2, http://links.lww.com/ALN/C15).

MicroRNA Expression

Several microRNAs that regulate autophagy-related genes and ubiquitin ligases were detected in the diaphragms of the spontaneously breathing group. Their relative abundances differed markedly; miR-206-3p was the most abundant, and miR-101a-3p was the least abundant (Supplemental Digital Content, fig. 3, http://links.lww.com/ALN/C15). There were also differences in the abundance of a given microRNA in the diaphragm and gastrocnemius muscles (Supplemental Digital Content, fig. 4, http://links.lww.com/ALN/C15). MiR-20a (0.56 ± 0.11-fold), miR-106b-5p (0.61 ± 0.11-fold), miR-376c-3p (0.36 ± 0.28-fold), miR-101a-3p (0.51 ± 0.32-fold), miR-204-5p (0.57 ± 0.28-fold), miR-93 to 5 (0.62 ± 0.12-fold), and miR-206-3p (0.61 ± 0.13-fold) decreased in the mechanical ventilation group, whereas miR-21-5p (2.70 ± 0.67-fold) increased, as compared with the spontaneously breathing group (fig. 5). No changes in microRNA expression were observed in the gastrocnemius muscle except for an increase in miR-210a-3p expression (2.41 ± 0.49-fold; fig. 6). *N*-Acetylcysteine administration had no effect on controlled

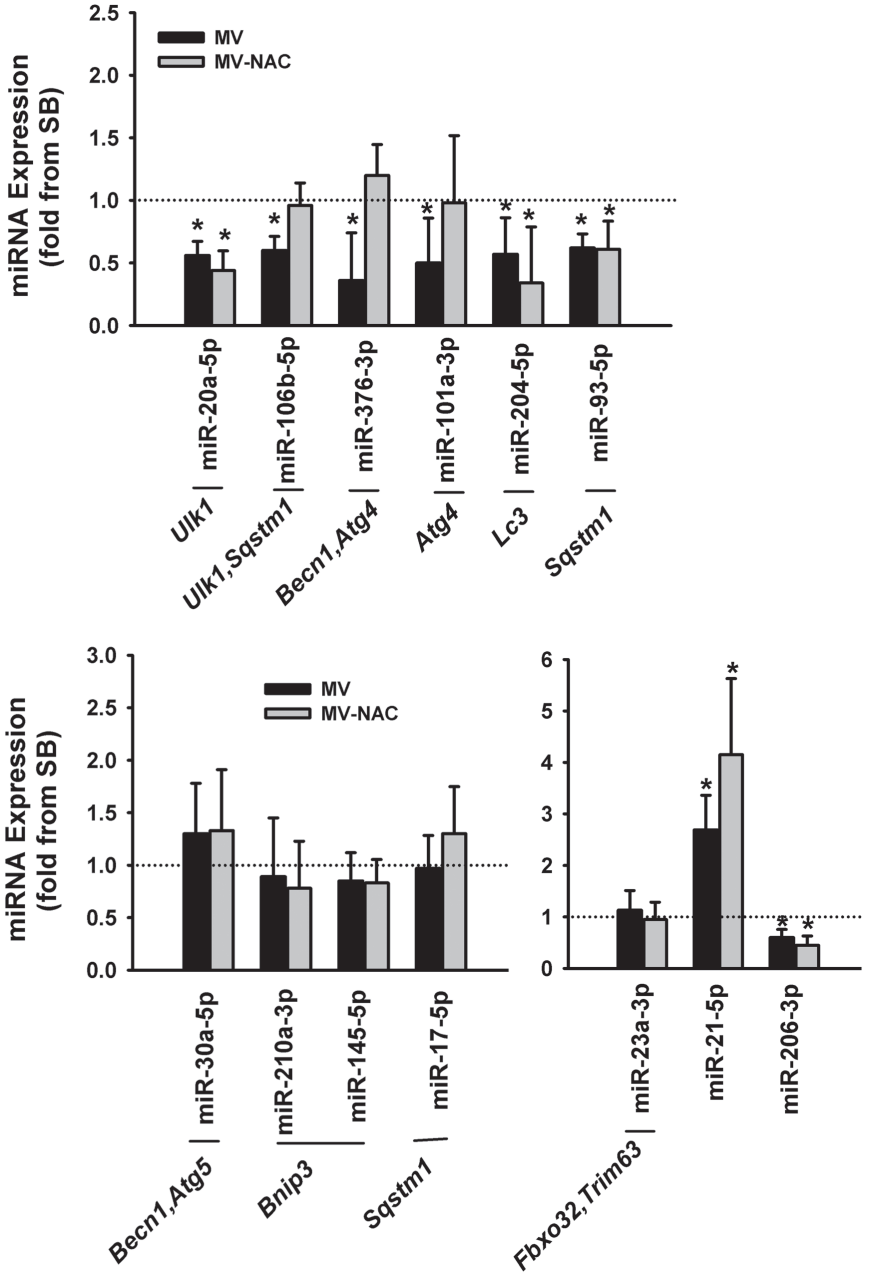

Fig. 5. MicroRNA expressions of various microRNAs that target autophagy-related genes and ubiquitin E3 ligases or that promote denervation-induced skeletal muscle atrophy (miR-21-5p and miR-206-3p) in diaphragms of the groups pretreated with saline before undergoing controlled mechanical ventilation for 24 h (MV) and pretreated with *N*-acetylcysteine before undergoing controlled mechanical ventilation for 24 h (MV-NAC; n = 6 per group), where n refers to the number of animals. The values are means ± SD, expressed as fold change from the group allowed to spontaneously breathe (SB; *dotted line*). *$P < 0.05$, compared with SB group. Comparisons between the three experimental groups were performed using one-way ANOVA.

mechanical ventilation–induced decreases in miR-20a-5p, miR-204-5p, miR-93-5p, and miR-206-3p but eliminated decreases in miR-106b-5p, miR-376c-3p, and miR-101a-3p (fig. 5). *N*-Acetylcysteine administration had no effect on the controlled mechanical ventilation–induced increase in miR-21-5p expression (fig. 5).

Myoblast Culture and Exposure to Hydrogen Peroxide

To evaluate whether autophagy-related genes and their corresponding microRNAs are regulated by reactive oxygen species, cultured C2C12 murine myoblasts were exposed for 24 h to phosphate-buffered saline (control) or hydrogen peroxide (100 μM). Exposure to hydrogen peroxide (H_2O_2) resulted

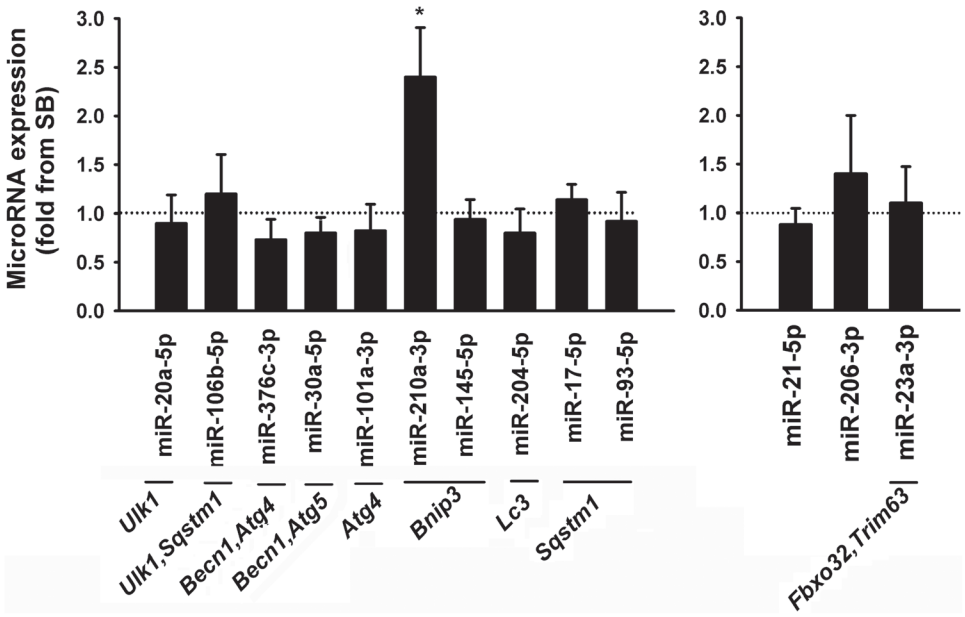

Fig. 6. Expression of various microRNAs in rat gastrocnemius of the group pretreated with saline before undergoing controlled mechanical ventilation for 24 h (MV). The values are the means ± SD, expressed as fold change from the group allowed to spontaneously breathe (SB; n = 9 per microRNA), where n refers to the number of animals. *$P < 0.05$, compared with the SB group. Comparisons between the three experimental groups were performed using one-way ANOVA.

in an increase in the intensity of LC3B-II protein; upregulation of the expressions of *Ulk1* (4.3 ± 0.44-fold), *Becn1* (2.91 ± 0.90-fold), *Atg4b* (1.86 ± 0.21-fold), *Bnip3* (2.11 ± 0.44-fold), *Lc3b* (5.52 ± 1.56-fold), and *Sqstm1* (9.91 ± 2.21-fold); and downregulation of the expressions of miR-106b-5p (0.56 ± 0.29-fold), miR-376-3p (0.47 ± 0.21-fold), miR-101a-3p (0.19 ± 0.16-fold), miR-204-5p (0.51 ± 0.23-fold), miR-210a-3p (0.57 ± 0.06-fold), and miR-145b-5p (0.48 ± 0.02-fold; fig. 7). Hydrogen peroxide exposure did not affect miR-20a-5p, miR-93-5p, miR-30a-5p, or miR-17-5p levels. We should emphasize that diaphragmatic levels of miR-20a-5p, miR-93-5p, miR-30a-5p, and miR-17-5p in the mechanical ventilation-NAC group remained unchanged after *N*-acetylcysteine administration, suggesting that they were unaffected by reactive oxygen species.

Discussion

This study demonstrates that prolonged controlled mechanical ventilation affects the diaphragm by decreasing contractility, increasing fiber atrophy, inducing the proteasome and autophagy pathways, and decreasing expressions of several microRNAs that negatively regulate autophagy genes. It also establishes that the antioxidant *N*-acetylcysteine significantly improves contractility, attenuates induction of the proteasome and autophagy pathways, and reverses decreases in the expressions of microRNAs that regulate autophagy genes.

Controlled Mechanical Ventilation–induced Diaphragm Weakness

Oxidative stress is responsible for controlled mechanical ventilation–induced diaphragm contractile dysfunction and fiber atrophy.[7,10,11,13,36] Our results indicating that administration of *N*-acetylcysteine prevents controlled mechanical ventilation–induced diaphragm weakness are in accordance with these prior observations. Excessive reactive oxygen species levels depress diaphragm contractility through direct oxidative damage to myofibrillar proteins, leading to decreased Ca^{2+} sensitivity.[37] High reactive oxygen species levels may also negatively affect diaphragm contractility during prolonged controlled mechanical ventilation through calpain activation, which leads to the degradation of cytoskeletal proteins that are crucial to the maintenance of sarcomere structure, resulting in sarcomere disruption and impairment of the muscle's ability to generate force.[38] Matecki *et al.*[39] have suggested that leaky ryanodine receptors contribute to muscle weakness during prolonged mechanical ventilation. They reported that oxidative stress and β-adrenergic signaling are responsible for the depressed contractility that is associated with an array of ryanodine receptors complex alterations (oxidation, *S*-nitrosylation, Ser^{2844} phosphorylation, and depletion of calstabin1) that occur in the diaphragm in response to prolonged controlled mechanical ventilation.

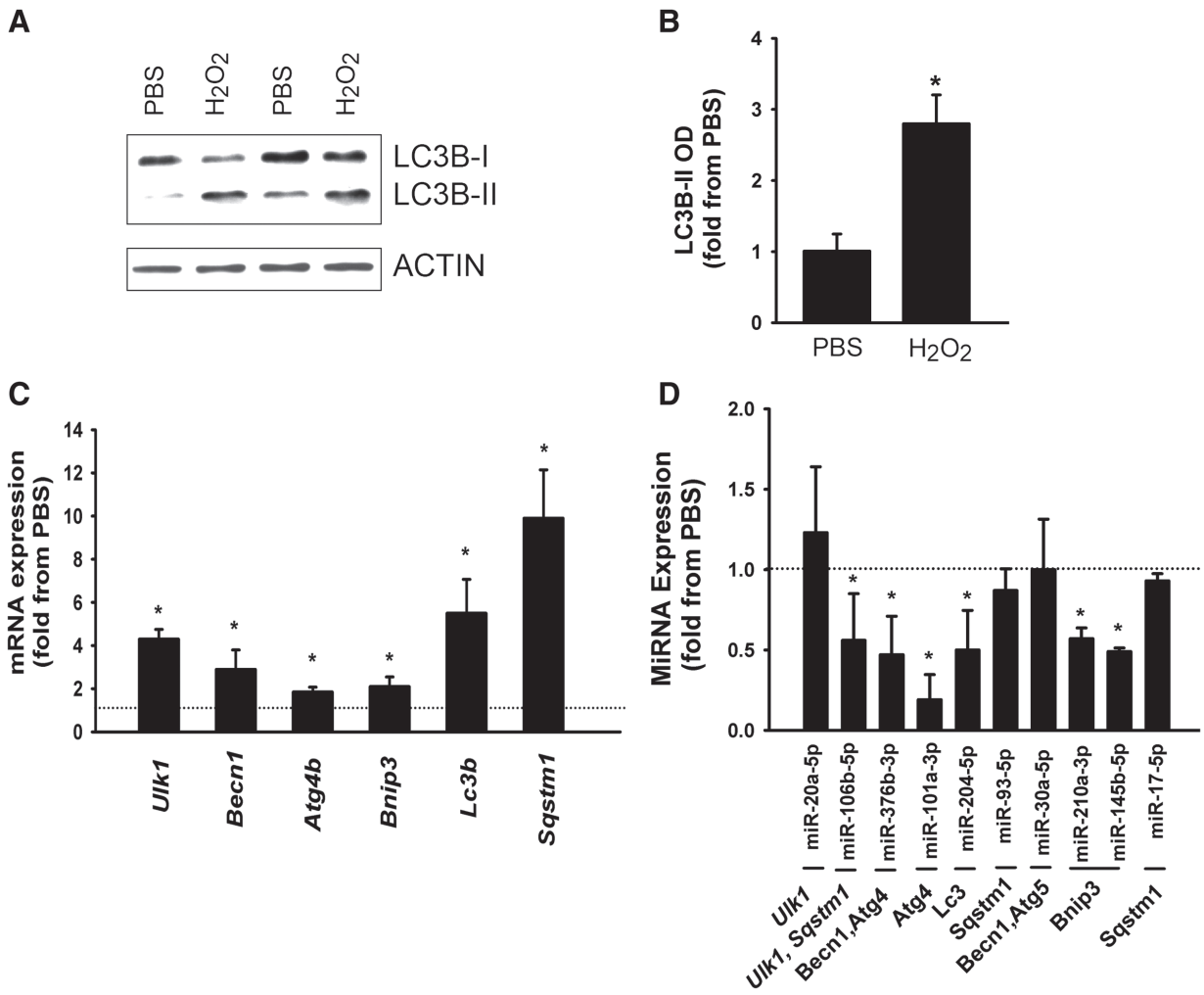

Fig. 7. Protein, mRNA, and microRNA (MiRNA) detection in C2C12 myoblasts exposed for 24 h to phosphate-buffered saline (PBS) or hydrogen peroxide (H_2O_2; 100 μM). (*A*) Representative immunoblots of LCB3 and ACTIN proteins. (*B*) Optical densities (OD) of LC3B-II protein intensity (n = 6/group). (*C*) mRNA expressions of *Ulk1*, *Becn1*, *Atg4b*, *Bnip3*, *Lc3b*, and *Sqstm1* (n = 6/group). (*D*) MicroRNA expressions of various microRNAs that target autophagy-related genes (n = 6/group). n refers to the number of cell culture wells. The values in (*C*) and (*D*) are means ± SD, expressed as fold change from PBS (*dotted lines*). *$P < 0.05$, compared with PBS. Comparisons between the three experimental groups were performed using one-way ANOVA.

Controlled Mechanical Ventilation–induced Proteasome Activation

We found that pretreatment with *N*-acetylcysteine attenuates controlled mechanical ventilation–induced protein ubiquitination and upregulation of *Fbxo32* and *Trim63* and eliminates *Foxo1* induction in the diaphragm. These findings affirm that controlled mechanical ventilation–induced proteasome activation in the diaphragm is redox-dependent. They also demonstrate that reactive oxygen species regulate the expression of *Foxo1* in the diaphragm during prolonged controlled mechanical ventilation but not that of *Foxo3*. Reactive oxygen species modulate FOXO transcriptional activities through posttranslational modifications, coregulator interactions, subcellular localization, and stability.[40]

Recent studies have revealed important roles for microRNAs in the regulation of proteolytic pathways in muscle fibers. For example, miR-23a suppresses the expression of both *Fbxo32* and *Trim63*[25], and miR-23a transgenic mice are resistant to glucocorticoid-induced skeletal muscle atrophy. In the current study, we found that miR-23a expression in the diaphragm was unaffected by prolonged controlled mechanical ventilation or *N*-acetylcysteine administration, suggesting that it was not responsible for the induction of *Fbxo32* and *Trim63* that was observed in the mechanical ventilation group.

In a previous study, our group demonstrated that *Fbxo30* expression is significantly induced in the limb muscles of septic mice.[32] *Fbxo30* expression is significantly induced in

the muscles of mice when bone morphogenetic protein signaling is inhibited, leading to muscle atrophy.[41] These results suggest that normal bone morphogenetic protein signaling inhibits *Fbxo30* expression. In the current study, *Fbxo30* expression in the diaphragm was unaffected by prolonged controlled mechanical ventilation but was attenuated by *N*-acetylcysteine, suggesting that reactive oxygen species are involved in the regulation of basal levels of *Fbxo30* possibly through Smad4 inhibition.[42]

Controlled Mechanical Ventilation–induced Autophagy Activation

We report here that prolonged controlled mechanical ventilation induces autophagy in the diaphragm, as evidenced by increased LC3B lipidation and the induction of several autophagy-related genes. These results are in agreement with previous studies in humans and animals undergoing prolonged controlled mechanical ventilation.[2,5,31] Without autophagic flux measurements, it remains unclear as to how *N*-acetylcysteine affects autophagy in the diaphragm during prolonged controlled mechanical ventilation. Based on reports that suggest that reactive oxygen species stimulate autophagy by activating HIF-1, FOXO, p53, and NRF2 transcription factors and through posttranscriptional events such as endoplasmic reticulum stress, oxidized protein accumulation, and mitochondrial dysfunction,[43,44] one interpretation could be that *N*-acetylcysteine attenuates autophagy by eliminating reactive oxygen species-induced stimulation of autophagosome formation. This theory is supported by our observations that *N*-acetylcysteine attenuates LC3B lipidation and decreases the expressions of several autophagy-related genes (fig. 4) and by the observations of Smuder *et al.*,[45] who recently showed that reactive oxygen species increase the expressions of several autophagy genes in the diaphragms of rats undergoing controlled mechanical ventilation for 12 h.

An alternative interpretation is that reactive oxygen species levels in the diaphragm increase in response to prolonged controlled mechanical ventilation and inhibit autophagy by specifically targeting autophagosome formation. *N*-Acetylcysteine administration restores autophagy back to control levels. This theory is supported by observations made in pulmonary epithelial cells that excessive reactive oxygen species levels inhibit autophagy.[46] It should be pointed out; however, that reactive oxygen species-induced inhibition of autophagy in that study was associated with significant decreases in LC3B lipidation and SQSTM1 protein accumulations. In our study, prolonged controlled mechanical ventilation triggered significant increases in LC3B lipidation and enhanced SQSTM1 transcription (fig. 4). Based on these findings, we suggest that increased reactive oxygen species levels stimulate rather than inhibit autophagy in the diaphragm in response to prolonged controlled mechanical ventilation. Future studies involving autophagic flux measurements are warranted to confirm this.

The role of autophagy in controlled mechanical ventilation–induced diaphragm weakness remains unclear. Smuder *et al.*[45] suggested that autophagy significantly contributes to the development of ventilator-induced diaphragm dysfunction through a positive feedback loop, whereby reactive oxygen species production stimulates autophagy in diaphragmatic muscle fibers, and this enhanced autophagy causes further reactive oxygen species production by selectively degrading antioxidant proteins such as catalase. Based on this theory, it is possible that the mechanism by which *N*-acetylcysteine administration prevents controlled mechanical ventilation–induced diaphragm weakness is by attenuating autophagy. It is also possible that enhanced autophagy is a compensatory adaptive response designed to recycle dysfunctional mitochondria and protein aggregates and that once reactive oxygen species levels are reduced by *N*-acetylcysteine, activation of autophagy is no longer necessary.

Recent studies suggest that autophagy is inhibited by specific microRNAs that selectively target genes that are involved in various steps of autophagosome formation and in mitophagy. For instance, ULK1 is targeted by miR-17, miR-20a, and miR-106b[15,22]; BECN1 is targeted by miR-376, miR-17, and miR-30a[16,17,23]; ATG4 is targeted by miR-101a and miR-376[16,18]; SQSTM1 is targeted by miR-17, miR-106b, and miR-93[24]; LC3 is targeted by miR-204[19]; and BNIP3 is targeted by miR-145 and miR-210a.[20,21] These microRNAs have not yet been detected in skeletal muscles. We found that miR-20a-5p, miR-106b-5p, miR-376-3p, miR-101a-3p, miR-204-5p, and miR-93-5p expressions decreased in the diaphragms of the mechanical ventilation group (fig. 5), whereas expressions of their targets (*Ulk1*, *Atg4b*, BECN1, LC3B, and SQSTM1) increased. It is notable that the decreases in microRNA expression that we observed were selective to the diaphragm, because they were not detected in the gastrocnemius muscle of the same group.

Our study also reveals that *N*-acetylcysteine administration reversed decreases in miR-106b-5p, miR-376-3p, and miR-101-3p expression, suggesting that endogenous reactive oxygen species exert a negative effect on this particular group of microRNAs. This was confirmed in C2C12 cells where exposure to hydrogen peroxide triggered significant downregulation of several microRNAs that regulate autophagy-related gene expression (fig. 7). The mechanisms through which reactive oxygen species regulate microRNA levels are complex and are involved in several steps of microRNA biogenesis, including transcription, DNA methylation, histone modification, and Drosha and Dicer enzyme processing.[47] Identification of the precise mechanisms through which *N*-acetylcysteine alters microRNA expression in the diaphragm is beyond the scope of this study, but it is clear that at least some microRNAs that target autophagy-related genes are reactive oxygen species–responsive. By inhibiting the production of reactive oxygen

species, *N*-acetylcysteine administration interferes with reactive oxygen species–microRNA regulatory interactions.

Soares *et al.*[26] reported that miR-21 and miR-206 induce muscle atrophy through selective targeting of transcription factor YY1 and the translation initiator factor eIF4E3. In the current study, we found that miR-21-5p expression increased and miR-206-3p expression decreased in the diaphragm of the mechanical ventilation group but not in the gastrocnemius. We also demonstrated that *N*-acetylcysteine administration had no effect on controlled mechanical ventilation–induced changes in miR-21-5p and miR-206 expressions in the diaphragm. These results suggest that enhanced miR-21-5p expression might have contributed to controlled mechanical ventilation–induced diaphragm atrophy.

Controlled Mechanical Ventilation–induced Diaphragm Atrophy

In our study, *N*-acetylcysteine administration did not prevent controlled mechanical ventilation–induced diaphragm fiber atrophy despite attenuation of the proteasomal and autophagic pathways (fig. 1). It has been reported that antioxidants such as trolox or the peptide SS-31 prevent controlled mechanical ventilation–induced diaphragm atrophy by inhibiting the calpain, caspase, and proteasome pathways.[7,9] In terms of the effects of antioxidants on controlled mechanical ventilation–induced diaphragm fiber atrophy, the differences between our study and those using trolox or SS-31 may be due to a number of factors, including the duration of controlled mechanical ventilation, the degree of diaphragm fiber atrophy induced by controlled mechanical ventilation, the nature of the antioxidants used, and the extent to which they inhibit proteolysis. It may be that *N*-acetylcysteine is not as efficient as trolox or SS-31 in attenuating mitochondrial reactive oxygen species. In our experiments, animals underwent 24 h of controlled mechanical ventilation. This duration triggers more severe diaphragm fiber atrophy than does 12 h of controlled mechanical ventilation, which was the duration used by other investigators. It is entirely possible that the preventative capacities of *N*-acetylcysteine were only partial in our study because the controlled mechanical ventilation–induced atrophy that developed was quite severe.

Clinical studies in critically ill patients have revealed that antioxidant therapies significantly decrease the rate of respiratory failure,[48] the duration of mechanical ventilation,[49,50] and the duration of intensive care unit stay.[50] Our study provides mechanistic insight into the ways in which antioxidants affect ventilatory muscle function under conditions of mechanical ventilation. We emphasize that antioxidant therapy may not affect the initial development of ventilator-induced diaphragm fiber atrophy but, when present, significantly improves diaphragm contractile performance, attenuates induction of the proteasome and autophagy pathways, and reverses downregulation of some microRNAs that regulate autophagy-related genes inside diaphragm muscle fibers.

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Competing Interests

The authors declare no competing interests.

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