

# Relationship between Autophagy and Ventilator-induced Diaphragmatic Dysfunction

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## ABSTRACT

**Background:** Mechanical ventilation (MV) is associated with atrophy and weakness of the diaphragm muscle, a condition termed ventilator-induced diaphragmatic dysfunction (VIDD). Autophagy is a lysosomally mediated proteolytic process that can be activated by oxidative stress, which has the potential to either mitigate or exacerbate VIDD. The primary goals of this study were to (1) determine the effects of MV on autophagy in the diaphragm and (2) evaluate the impact of antioxidant therapy on autophagy induction and MV-induced diaphragmatic weakness.

**Methods:** Mice were assigned to control (CTRL), MV (for 6 h), MV + *N*-acetylcysteine, MV + rapamycin, and prolonged (48 h) fasting groups. Autophagy was monitored by quantifying (1) autophagic vesicles by transmission electron microscopy, (2) messenger RNA levels of autophagy-related genes, and (3) the autophagosome marker protein LC3B-II, with and without administration of colchicine to calculate the indices of relative autophagosome formation and degradation. Force production by mouse diaphragms was determined *ex vivo*.

**Results:** Diaphragms exhibited a 2.2-fold (95% CI, 1.8 to 2.5) increase in autophagic vesicles visualized by transmission electron microscopy relative to CTRL after 6 h of MV ( $n = 5$  per group). The autophagosome formation index increased in the diaphragm alone (1.5-fold; 95% CI, 1.3 to 1.8;  $n = 8$  per group) during MV, whereas prolonged fasting induced autophagosome formation in both the diaphragm (2.5-fold; 95% CI, 2.2 to 2.8) and the limb muscle (4.1-fold; 95% CI, 1.8 to 6.5). The antioxidant *N*-acetylcysteine further augmented the autophagosome formation in the diaphragm during MV (1.4-fold; 95% CI, 1.2 to 1.5;  $n = 8$  per group) and prevented MV-induced diaphragmatic weakness. Treatment with the autophagy-inducing agent rapamycin also largely prevented the diaphragmatic force loss associated with MV ( $n = 6$  per group).

**Conclusions:** In this model of VIDD, autophagy is induced by MV but is not responsible for diaphragmatic weakness. The authors propose that autophagy may instead be a beneficial adaptive response that can potentially be exploited for therapy of VIDD. (*ANESTHESIOLOGY* 2015; 122:1349-61)

MECHANICAL ventilation (MV) can be life saving but is also associated with adverse effects on the diaphragm muscle, a condition termed ventilator-induced diaphragmatic dysfunction (VIDD).<sup>1</sup> Because diaphragmatic function is a critical factor in successful weaning from MV, VIDD has the potential to increase the length of time patients require MV, resulting in an escalation of complications and costs. VIDD is characterized by diaphragmatic weakness resulting from both fiber atrophy and an impairment of the intrinsic force-generating capacity (specific force) of the muscle.<sup>2,3</sup> The loss of specific force in the diaphragm occurs early, before the onset of significant atrophy.<sup>4</sup> Although the cellular mechanisms responsible for this weakness remain poorly understood, a consistent finding in human and animal studies is the presence of increased oxidative stress in the diaphragm.<sup>5-8</sup> In animal models, antioxidants can prevent both specific force loss

### What We Already Know about This Topic

- Mechanical ventilation is associated with atrophy and weakness of the diaphragm muscle, called ventilator-induced diaphragmatic dysfunction

### What This Article Tells Us That Is New

- Autophagy was rapidly induced in the diaphragm in a mouse model of ventilator-induced diaphragmatic dysfunction, but the autophagic process was not responsible for diaphragmatic weakness

and atrophy of the diaphragm during MV.<sup>8-11</sup> Furthermore, dysfunctional or damaged mitochondria appear to be the main source of oxidative stress in this setting.<sup>8,12,13</sup>

Autophagy ("self-eating") is a catabolic process characterized by the formation of specialized vesicles

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(autophagosomes) that engulf cytoplasmic elements and then fuse with lysosomes to degrade their contents.<sup>14,15</sup> It serves to augment cellular energy production under nutrient deprivation conditions and also plays a critical role in cellular homeostasis by eliminating dysfunctional proteins and organelles, including damaged mitochondria (termed mitophagy).<sup>16</sup> Without autophagy, cells are potentially exposed to the harmful effects of disordered mitochondrial reactive oxygen species generation. Previous studies by our group<sup>7</sup> and others<sup>17,18</sup> have provided the evidence for up-regulation of the autophagy pathway in the diaphragm during MV, principally by demonstrating increased levels of the autophagosome marker, microtubule-associated protein light chain 3 (LC3B)-II.

However, it is important to recognize that increased LC3B-II levels can represent a reduction in autophagosome degradation (*i.e.*, reduced “autophagic flux”) rather than actual induction of the autophagy pathway.<sup>19</sup> This has been postulated in nonrespiratory muscles of critically ill patients<sup>20</sup> and has been implicated as a cause of organ dysfunction in sepsis.<sup>21</sup> In addition, previous studies did not control for the potential confounding effects of reduced nutritional intake during MV, which could also account for autophagy induction under these conditions. Therefore, in the current study, our first hypothesis was that the accumulation of LC3B-II in the diaphragm during MV could be primarily due to a pathological impairment of autophagosome degradation rather than up-regulation of the autophagy pathway. If this was the case, the nature of autophagosome dynamics (autophagosome production *vs.* degradation) should differ greatly between MV and the classical autophagy stimulus of prolonged fasting, as in the latter situation, both autophagosome production and degradation are greatly accelerated.<sup>22,23</sup>

Furthermore, the physiological relevance of altered autophagy regulation to the loss of diaphragmatic force-generating capacity observed during MV remains to be established. Antioxidant treatment is able to prevent the development of diaphragmatic weakness during MV in animal models. However, it is unknown whether this benefit is linked to effects on autophagy. Although it has been found that oxidative stress can be a potent stimulus for the induction of autophagy,<sup>24,25</sup> most of the data on this point have been derived from *in vitro* studies. Given our first hypothesis that autophagic flux in the diaphragm could be impaired by the use of MV, our second hypothesis was that antioxidant therapy does not exert its mitigating effects on VIDD by further inhibiting autophagy. Rather, we postulated that active stimulation of autophagy during MV might be beneficial and thus help to preserve diaphragmatic force under these conditions.

Accordingly, our specific objectives in this study were to (1) examine the autophagosome dynamics in the diaphragm and limb musculature during MV; (2) compare the nature and magnitude of changes in autophagosome dynamics between MV and the classical autophagy stimulus of

prolonged fasting; (3) ascertain whether the improvement in diaphragmatic force production during MV observed with antioxidant therapy is associated with modulation of autophagy in the diaphragm; and (4) determine whether pharmacological stimulation of autophagy has an impact upon the level of diaphragmatic force loss induced by MV.

## Materials and Methods

### Animals

Male mice (C57BL/6) 8 to 10 weeks of age and weighing approximately 25 g (Charles River Laboratories, Canada) were used. The mice were housed under a standard alternating 12-h light–dark cycle with food and water provided *ad libitum* before the experiments. During experiments, all fasted mice had continuous access to water. The study was approved by the local institutional animal ethics committee of the McGill University Health Centre (Canada) in accordance with Canadian Council on Animal Care guidelines.

Mice were randomly divided into the following experimental groups (the number of animals per group in each experiment can be found in the corresponding figure legends): (1) mechanically ventilated (MV group), subjected to controlled MV for 6 consecutive hours under continuous anesthesia (see section on Mechanical Ventilation for further details); (2) nonventilated control mice (CTRL group), all of which were maintained without access to food for 6 h before euthanasia to match their nutritional intake to the MV group; (3) prolonged fasting (fasting group), without access to food for a period of 48 h before euthanasia; (4) mechanically ventilated mice treated with the antioxidant agent *N*-acetylcysteine (NAC) (MV + NAC group), administered by intraperitoneal injection at a dose of 200 mg/kg (Sigma-Aldrich A7250, Canada; dissolved in phosphate-buffered saline with pH adjusted to 7.4) immediately after intubation and the initiation of MV<sup>26</sup>; and (5) mechanically ventilated mice treated with the autophagy-inducing agent rapamycin (MV + RAPA group) delivered by intraperitoneal injection at a dose of 10 mg/kg (LC Laboratories, USA; dissolved in vehicle consisting of 5% PEG-400, 5% Tween-80, and 4% ethanol) on 3 consecutive days including the day of MV (3 h before)<sup>27</sup>; for these last experiments, a separate MV group underwent the same protocol injected with the vehicle only. For the majority of experiments, a subset of mice within each experimental group was treated with colchicine (+COL) to evaluate autophagosome dynamics (see section on Autophagosome Dynamics for further details). Upon completion of the experiments, the diaphragm and, in some cases, the extensor digitorum longus (EDL, a fast-twitch hindlimb muscle) were harvested.

### Autophagosome Dynamics

The abundance of autophagosomes within a tissue reflects the balance between autophagosome formation and autophagosome degradation by the lysosomal compartment.<sup>19,28</sup> Colchicine and other agents that block autophagosome degradation

by the lysosome are frequently used to evaluate the dynamics of this process.<sup>19</sup> For this purpose, mice were treated with colchicine (Sigma-Aldrich C9754) at 48 and 24 h before animal sacrifice at a dose of 0.4 mg/kg by intraperitoneal injection as in previous studies.<sup>25,27,29</sup> The LC3B-II protein levels observed in colchicine-treated groups (*i.e.*, colchicine-treated values from different experimental groups compared with each other) were used to infer different rates of autophagosome formation based on the assumption of equal autophagosome degradation blockade across groups under these conditions. The magnitude of change in LC3B-II protein levels induced by colchicine treatment (*i.e.*, the difference between colchicine-treated and colchicine-untreated values) was used as an index of the autophagosome degradation rate as previously described.<sup>19</sup>

### LC3B Immunoblotting

Frozen muscle tissues were homogenized in lysis buffer supplemented with protease inhibitors and centrifuged, followed by determination of protein concentration on the supernatant using a Bradford assay. Forty microgram of muscle lysate protein per lane was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membrane for LC3B (Cell Signaling 3868, USA) immunoblotting. The dilutions of primary and secondary antibodies were according to the manufacturer's instructions. Quantification of protein bands was performed by electrochemiluminescence and the Odyssey<sup>®</sup> Infrared Imaging System (LI-COR<sup>®</sup> Biosciences, USA) by using Ponceau red staining to adjust for any variations in protein loading.

### Electron Microscopy

Transmission electron microscopy was used to examine and quantify autophagic structures. Immediately after euthanasia, the diaphragm was dissected into approximately 5-mm wide strips of muscle and fixed by immersion in a 2% glutaraldehyde solution in 0.1 M cacodylate buffer, pH 7.4, as previously described in detail.<sup>30</sup> Diaphragm strips were cut into smaller segments and embedded in resin at 60°C for 24 h. Ultrathin sections of 70 nm were cut using a diamond knife on a Leica EM UC7 ultramicrotome. Sections were stretched and mounted on Pioloform filmed copper grids before staining with 2% aqueous uranyl acetate and lead citrate (Leica, Germany). Ultrathin sections were examined on a Phillips CM 100 Compustage transmission electron microscope (Agar Scientific, United Kingdom), and digital micrographs were captured by an AMT CCD camera (Deben, United Kingdom). Autophagic structures consisting of double-membrane autophagosomes and/or abnormally electron-dense lysosomal vesicles were quantified by a blinded observer in 59 (CTRL group) and 57 (MV group) regions of interest (mean area = 60.5  $\mu\text{m}^2$ ), defined as areas containing a high mitochondrial density.

### Real-time Quantitative Polymerase Chain Reaction

Total RNA was extracted from frozen muscle tissues using TRIZOL reagent (Invitrogen, U.S.A.). The isolated RNA

was treated with DNase I, followed by further purification using the RNeasy mini kit (Qiagen, Canada) according to the manufacturer's instructions. Real-time polymerase chain reaction (PCR) was performed by using a StepOne Plus Real-Time PCR System (Applied Biosystems, USA). Specific primers were designed to detect the expression of several autophagy-related genes as shown in table 1. PCR amplification was carried out for 40 cycles at a melting temperature of 95°C for 15 s and an annealing temperature of 60°C for 60 s. A dissociation curve was analyzed for each PCR experiment to assess primer–dimer formation or contamination. Relative messenger RNA (mRNA) level quantifications of target genes in the MV group were determined using the cycle threshold method with hypoxanthine phosphoribosyltransferase 1 as the housekeeping gene, and the data were expressed as fold-change compared with the CTRL group.

### Diaphragm Contractility

Contractile force measurements of the diaphragm were performed as previously described by an operator blinded to sample identity.<sup>4,8</sup> In brief, the diaphragm muscle was surgically excised immediately after animal sacrifice and placed into a chilled (4°C) and equilibrated (95% O<sub>2</sub>–5%CO<sub>2</sub>, pH 7.38) Krebs solution. A muscle strip about 2 mm wide was dissected free, taking care to leave the central tendon and adjacent rib cage margins intact. The excised diaphragm strip was mounted into a jacketed tissue bath chamber filled with Krebs solution by using a custom-built muscle holder containing two stimulation electrodes located on either side. A thermoequilibration period of 15 min was observed before initiating contractile measurements at 23°C. After placing the diaphragm strip at optimal length, the force–frequency relationship was determined by sequential supramaximal stimulation for 1 s at 10, 30, 50, 100, and 150 Hz, with 2 min between each stimulation train. The force data were acquired to computer at a sampling rate of 1,000 Hz for later analysis. After completion of the above contractility studies, the muscles were removed from their baths, and muscle length was measured with a microcaliper accurate to 0.1 mm. Muscle force was normalized to tissue cross-sectional area, which was determined by assuming a muscle density of 1.056 g/cm<sup>3</sup>. Specific force (force/cross-sectional area) is expressed in newtons per square centimeter.

**Table 1.** Primer Sequences for Real-time Polymerase Chain Reaction Quantification of Autophagy-related Gene Transcripts

Gene	Sequence
BNIP3	Forward: CTGGACGAAGTAGCTCCA Reverse: ACAGAGTGCTGTTTTCT
LC3B	Forward: CGATACAAGGGGGAGAAGCA Reverse: ACTTCGGAGATGGGAGTGGA
GABARAPL1	Forward: GAAGGCTCCTAAAGCCAGGG Reverse: TAAGGCGTCTCAGGTCTCA

### Mechanical Ventilation

Mechanical ventilation group mice received 6 h of controlled MV (*i.e.*, with minimal spontaneous respiratory effort) using a previously described protocol.<sup>4</sup> A duration of MV of 6 h was selected based on previous work showing a significant reduction in diaphragmatic force-generating capacity.<sup>4</sup> In brief, MV mice were anesthetized by intraperitoneal injection of xylazine (10 mg/kg body weight) and pentobarbital sodium (50 mg/kg body weight) and then orally intubated. No neuromuscular-blocking agent was used. The endotracheal tube was connected to a small animal ventilator (Minivent®; Harvard Apparatus, Canada) with the following settings: fraction of inspired oxygen of 0.21 (room air), controlled volume mode with tidal volume of 10  $\mu$ l/mg body weight, respiratory rate of 150 to 170 breaths/min, and positive end-expiratory pressure level of 3 to 4 cm H<sub>2</sub>O achieved by placing the expiratory port under a water seal. Animals were placed on a prewarmed homeothermic blanket (Homeothermic Blanket Control Unit; Harvard Apparatus), and the mice received hourly intraperitoneal injections of lactated Ringer's solution (0.10 ml) to compensate insensible losses. In addition, pentobarbital was administered every 1 to 1.5 h to maintain adequate anesthesia as indicated by lack of animal movement or spontaneous respiratory efforts.

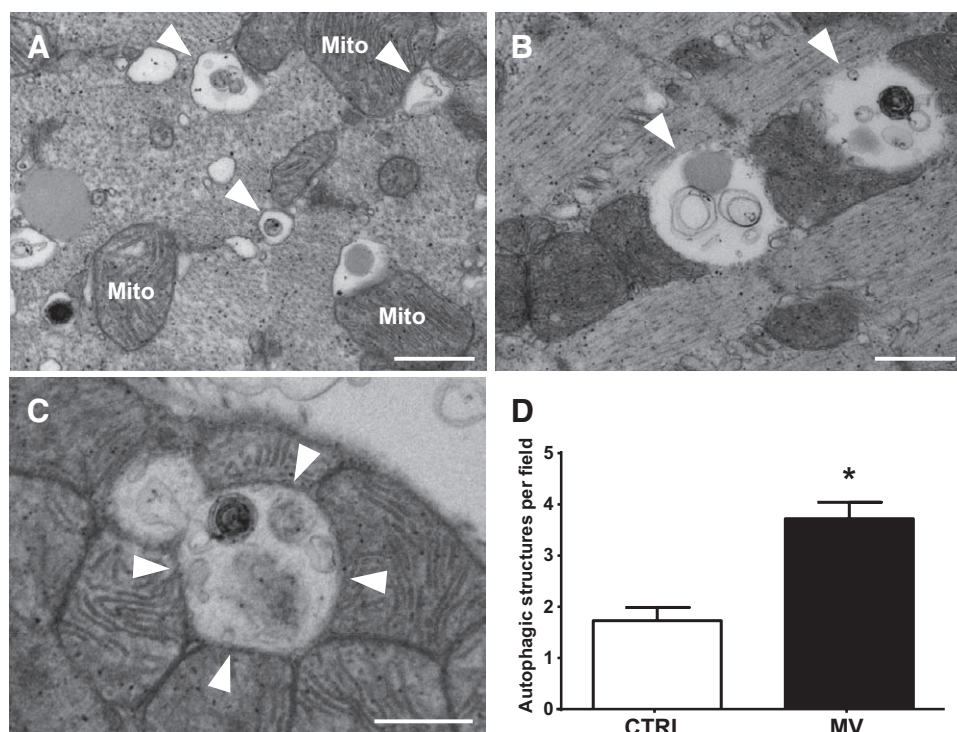
### Statistical Analysis

All data are expressed as means  $\pm$  SEM, together with 95% CIs (see figure legends). Statistical differences between two groups were analyzed by using a standard two-tailed *t* test (assuming unpaired data sets and unequal variances). When more than two groups were compared, ANOVA (either one or two way) was performed followed by *post hoc* application of the Tukey method when appropriate. The  $\alpha$  level was set at 0.05 for all tests (GraphPad Prism, USA). The number of mice 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.

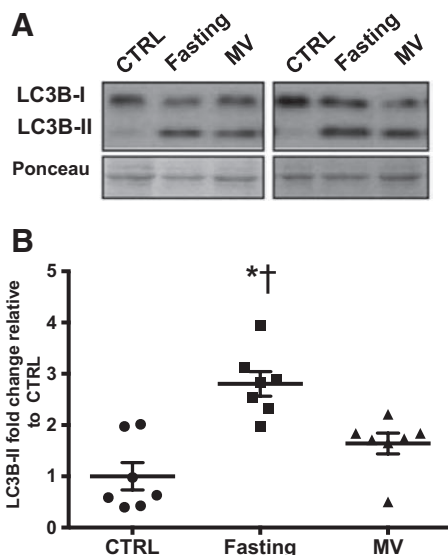
### Results

#### Autophagic Structures Are Increased in MV Diaphragms

The most traditional method for monitoring autophagic structures is by electron microscopy. As shown in figure 1, A–C, transmission electron microscopy revealed vesicular structures enclosing cytoplasmic cargo consistent with autophagosomes in MV group diaphragms. These contained a variety of undigested cytoplasmic contents, some of which appeared to represent organelles such as mitochondria. When the number of such autophagic structures was quantified in a blinded manner, a significant increase of



**Fig. 1.** Autophagosome numbers are increased in diaphragms of mechanically ventilated (MV) mice. (A–C) Representative electron microscopy images showing autophagosomes containing heterogeneous cargo (white arrowheads) in the diaphragms of MV mice; Mito = mitochondrion; scale bar = 500 nm. (D) Quantification of autophagic structures in control (CTRL; mean, 1.7; 95% CI, 1.2 to 2.2) versus MV (mean, 3.7; 95% CI, 3.1 to 4.4) diaphragms. \**P* < 0.05 versus CTRL (unpaired *t* test, *n* = 5 mice per group).



**Fig. 2.** Steady-state LC3B-II levels in diaphragms of mechanically ventilated (MV) mice. (A) Immunoblot images showing LC3B protein levels in control (CTRL), fasting (48 h), and MV group diaphragms. (B) Quantification of LC3B-II levels (normalized to Ponceau) in fasting (mean, 2.8; 95% CI, 2.2 to 3.4) and MV (mean, 1.6; 95% CI, 1.1 to 2.1) diaphragms, expressed as fold-change relative to average CTRL value (mean, 1.0; 95% CI, 0.3 to 1.7). \* $P < 0.05$  versus CTRL; † $P < 0.05$  versus MV (ANOVA,  $n = 7$  mice per group).

approximately 2.2-fold was observed in the diaphragms of mice subjected to 6 h of MV relative to nonventilated CTRL group diaphragms (fig. 1D).

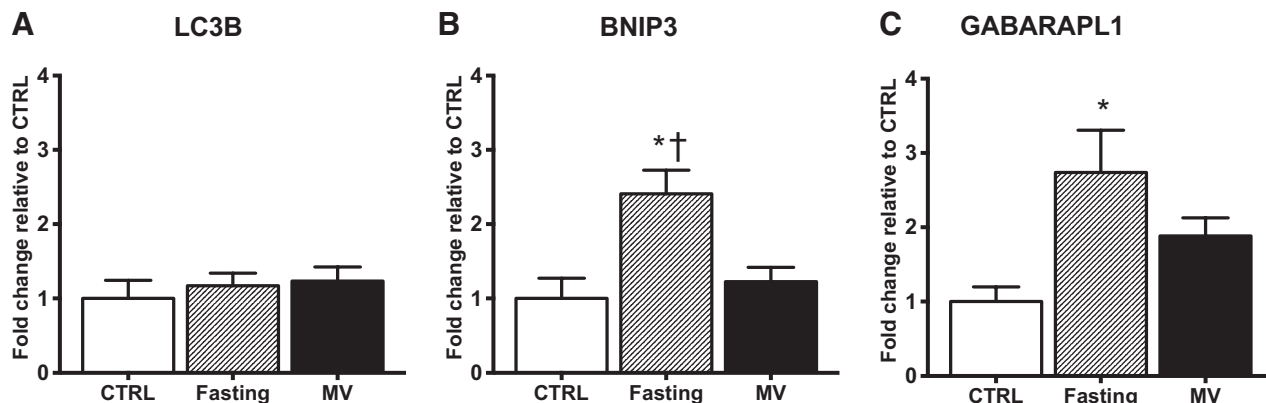
The lipidated form of LC3B (LC3B-II) is a biochemical marker of autophagosomes. In keeping with the ultrastructural data, there was an approximately 1.6-fold increase in the levels of LC3B-II in MV group diaphragms relative to CTRL mice whose food intake was equivalently restricted (fig. 2, A and B) although this difference did not reach

statistical significance. We also made comparisons to the classical autophagy stimulus of prolonged caloric restriction by fasting another group of mice for 48 h. This fasting group showed significant increases in LC3B-II in the diaphragm compared with both CTRL and MV mice. Taken together, these data suggest that short-term MV (6 h) and especially prolonged fasting (48 h) induce autophagosome accumulation within the mouse diaphragm.

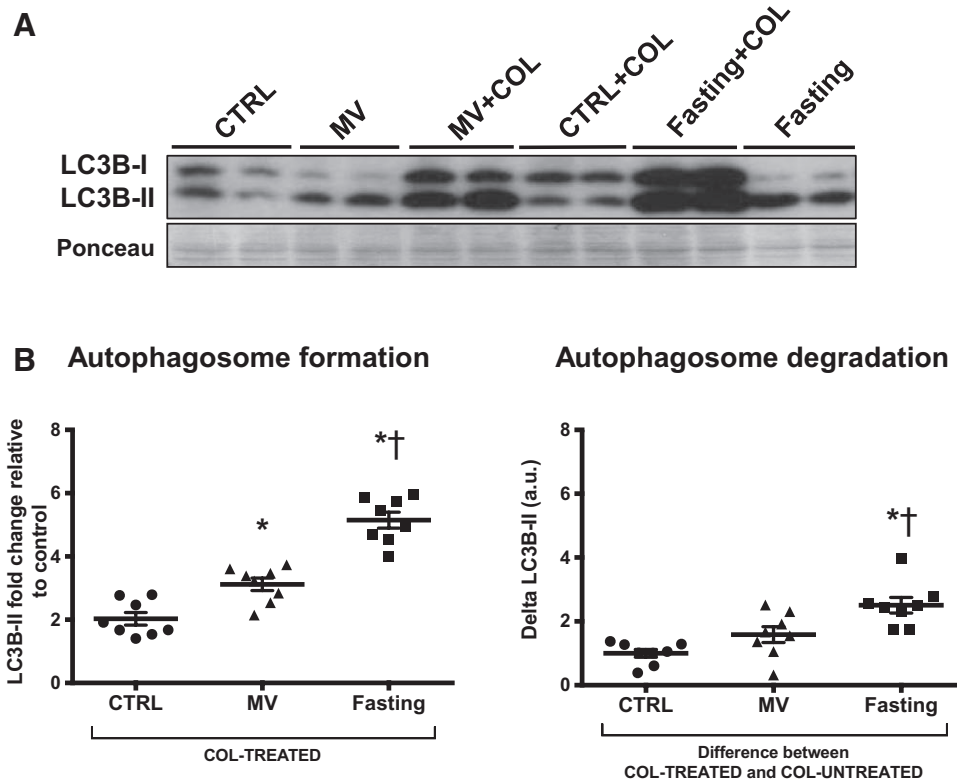
### **Autophagy Is Increased in Diaphragm but Not Limb Muscle during MV**

An accumulation of autophagosomes is not necessarily an indication of increased autophagy pathway induction and may in fact represent an inhibition of autophagic flux caused by impaired autophagosome degradation. To determine the cause of autophagosome accumulation in the diaphragm during MV, we first compared mRNA expression levels of prototypical autophagy-related genes (LC3B, BNIP3, and GABARAPL1) between CTRL, MV, and fasting group diaphragms (fig. 3). Of the genes tested, BNIP3 and GABARAPL1 demonstrated significant increases over CTRL values in the fasting group. A similar trend was observed in the MV group with GABARAPL1 although it did not reach statistical significance.

To more directly address the question of whether an increase in autophagosome formation is induced by MV, mice were treated with the microtubule-disrupting agent colchicine to block downstream degradation of autophagosomes by the lysosomal system (fig. 4A). Among colchicine-treated mice, there were increased LC3B-II levels in the MV group and even greater increases in the fasting mice relative to the CTRL group, consistent with an increased rate of autophagosome formation in the former two groups (fig. 4B). Furthermore, the change in LC3B-II levels between colchicine-treated and colchicine-untreated mice within each cohort (reflecting the autophagosome degradation rate)



**Fig. 3.** Autophagy-related gene transcripts in the diaphragm during mechanical ventilation (MV). Quantification of messenger RNA (mRNA) transcript levels for prototypical autophagy-related genes, expressed as fold-change relative to average control (CTRL) value (normalized to HPRT1). For BNIP3, mRNA levels in the fasting group (mean, 2.4; 95% CI, 1.7 to 3.2) were increased relative to MV (mean, 1.2; 95% CI, 0.8 to 1.7) and CTRL (mean, 1.0; 95% CI, 0.4 to 1.6). For GABARAPL1, mRNA levels were increased in the fasting group (mean, 2.7; 95% CI, 1.4 to 4.1) relative to CTRL (mean, 1.0; 95% CI, 0.5 to 1.5) but not MV (mean, 1.9; 95% CI, 1.3 to 2.5). \* $P < 0.05$  versus CTRL; † $P < 0.05$  versus MV (ANOVA,  $n = 8$  mice per group).



**Fig. 4.** Autophagosome formation is induced by mechanical ventilation (MV) in the diaphragm. (A) Representative immunoblots used for quantification of LC3B-II levels (normalized to Ponceau) in either the absence or presence (+COL) of previous colchicine administration to block autophagosome degradation. (B) *Left panel:* Comparisons of LC3B-II levels between colchicine-treated mice (expressed as fold-change relative to mean value in control mice without colchicine) to assess autophagosome formation. Among animals treated with colchicine, the MV group had increased levels of LC3B-II (mean, 3.1; 95% CI, 2.7 to 3.6) compared with the control (CTRL) group (mean, 2.0; 95% CI, 1.6 to 2.5), whereas the fasting group values (mean, 5.1; 95% CI, 4.5 to 5.7) exceeded both CTRL and MV. *Right panel:* Comparisons of the change (delta) in LC3B-II levels induced by colchicine within each experimental cohort to assess the autophagosome degradation. The average difference between colchicine-treated and colchicine-untreated values within each group was greater in the fasting group (mean, 2.5; 95% CI, 1.9 to 3.1) than in the MV (mean, 1.6; 95% CI, 1.0 to 2.2) or CTRL (mean, 1.0; 95% CI, 0.7 to 1.3) groups. \* $P < 0.05$  versus CTRL; † $P < 0.05$  versus MV (ANOVA,  $n = 8$  mice per group). COL = colchicine.

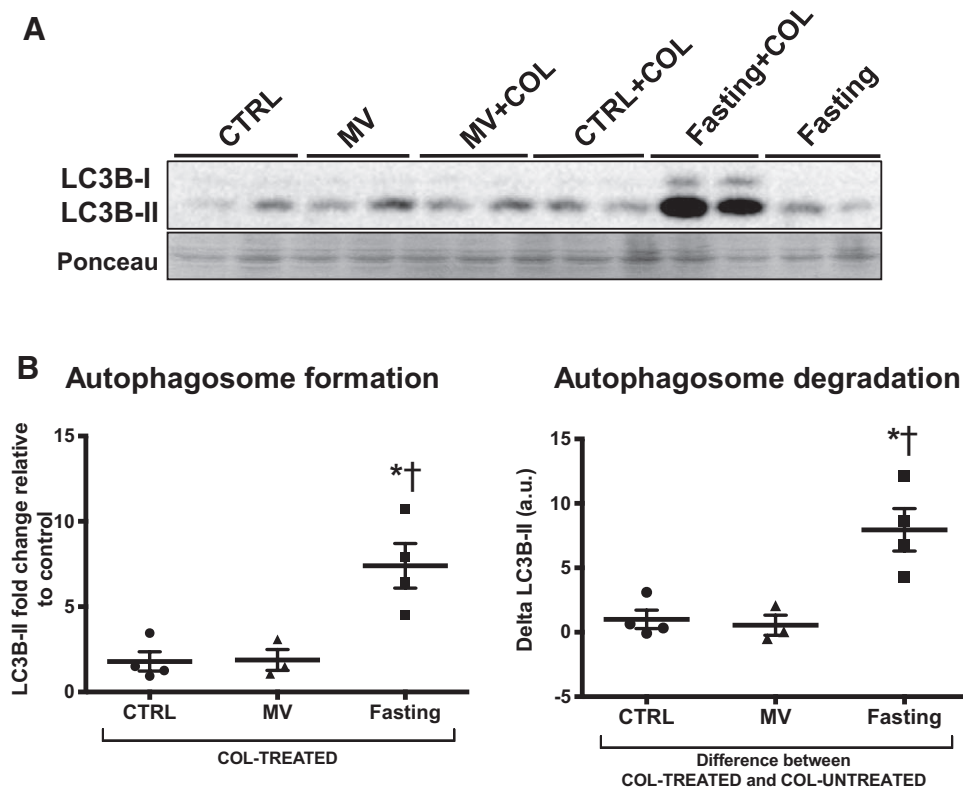
also tended to be greater in the MV group and was significantly increased in the fasting mice (fig. 4B). Taken together, these findings are in keeping with an increase of autophagy pathway activation in the MV and fasting groups relative to CTRL in the diaphragm muscle.

To determine whether the effects of MV upon autophagic activity in the diaphragm are specific to respiratory muscle, we also studied the EDL in a subset of mice (fig. 5A); this particular hindlimb muscle was selected because it has been shown to maintain normal force production during MV in this model.<sup>4</sup> In contrast to the diaphragm, the EDL did not demonstrate differences in LC3B-II levels between the CTRL and MV groups in colchicine-treated mice (fig. 5B). This indicates that, in contrast to the diaphragm, autophagosome formation was not increased by MV in the EDL. Similarly, the change in LC3B-II levels between colchicine-treated and colchicine-untreated mice was not altered by MV in the EDL, suggesting no change in autophagosome degradation rate in this muscle as a result of MV *per se*

(fig. 5B). In the prolonged fasting group, however, large increases in LC3B-II levels were observed in colchicine-treated mice, resulting in a significant change in LC3B-II levels between colchicine-treated and colchicine-untreated mice. These findings indicate that the rates of autophagosome production as well as degradation were both greatly increased in the EDL after 48 h of fasting.

#### Relationship of Autophagy to MV-induced Force Loss in the Diaphragm

Previous work in a rat model of VIDD has demonstrated that administration of the antioxidant NAC is able to prevent MV-induced force loss in the diaphragm,<sup>10</sup> but the relationship of antioxidant treatment to autophagic activity in the diaphragm under these conditions has not been determined. As shown in figure 6A, administration of NAC did not significantly affect the mRNA transcript levels of LC3B, BNIP3, or GABARAPL1 in diaphragms of mice undergoing MV. However, when mice were treated with colchicine



**Fig. 5.** Autophagy is not induced by mechanical ventilation (MV) in the hindlimb muscle. (A) Representative immunoblots of the extensor digitorum longus (EDL) muscle used for quantification of LC3B-II levels (normalized to Ponceau) in either the absence or presence (+COL) of previous colchicine administration. (B) The same analysis as described in figure 4B was used to evaluate effects of MV or fasting on autophagosome formation and degradation in the EDL. Note that MV had no significant impact upon these parameters in the EDL, whereas large effects upon autophagosome dynamics were observed in the EDL with prolonged fasting. \* $P < 0.05$  versus control (CTRL); † $P < 0.05$  versus MV (ANOVA,  $n = 3$  to 4 mice per group). COL = colchicine.

to probe autophagosome dynamics (fig. 6B), this revealed that autophagosome formation in the diaphragm, but not autophagosome degradation, was significantly increased by the use of NAC in mechanically ventilated mice (fig. 6C). Taken together, these data indicate that autophagy pathway activation during MV is not inhibited and actually appears to be further augmented by NAC administration.

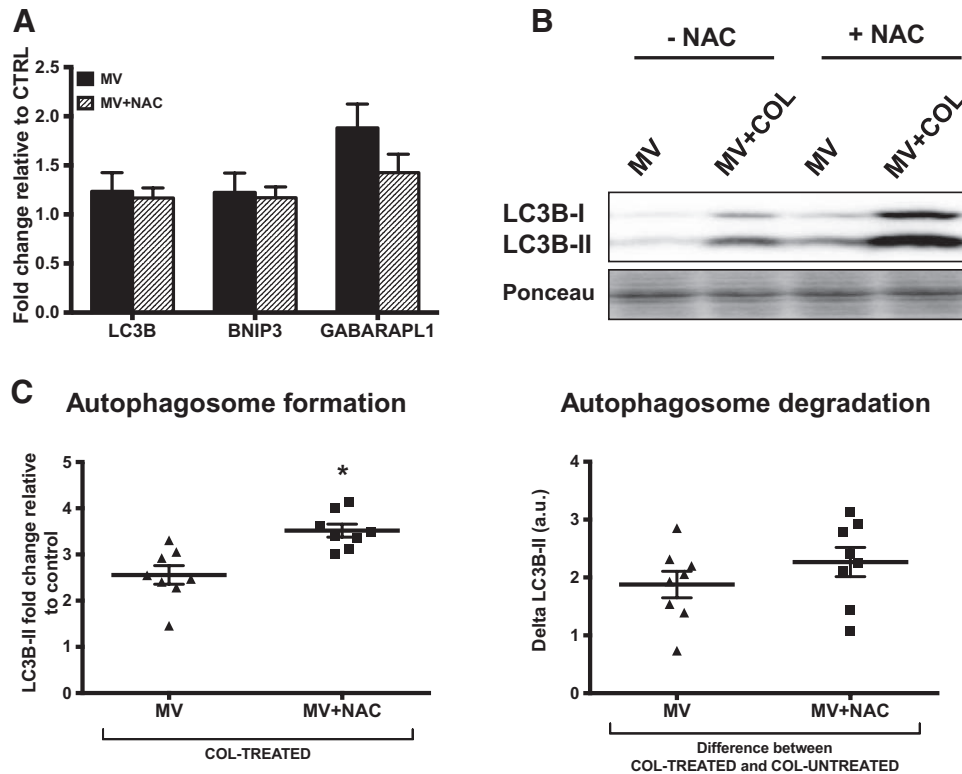
To determine the relationship of these findings to diaphragmatic force loss during MV, we verified that NAC does indeed have beneficial effects upon diaphragmatic force production in our murine model of VIDD. As depicted by the force–frequency curves in figure 7A, force-generating capacity of the diaphragm was significantly reduced in the MV group in comparison with CTRL values, with a decrease in maximum specific force of approximately 30%. In contrast, mice injected with a single dose of NAC immediately before the onset of MV exhibited no reduction in diaphragmatic strength compared with CTRL animals. Hence, NAC completely prevented the MV-induced diaphragmatic force loss.

To further examine the interaction between autophagy and diaphragmatic force-generating capacity during MV, mice were treated with the classical autophagy-inducing agent rapamycin before MV, which increased LC3B-II levels in the diaphragm by 64% ( $P = 0.014$ ) compared with

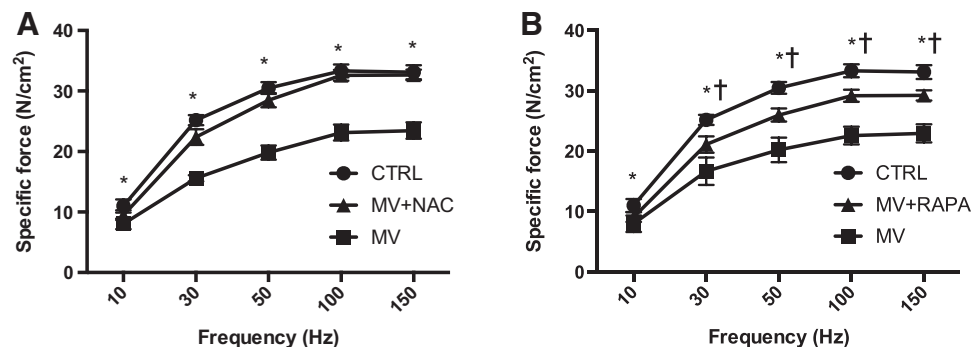
vehicle-treated mice after 6 h of MV. As shown in figure 7B, rapamycin treatment was associated with significant improvements in force-generating capacity of the diaphragm at most stimulation frequencies. Therefore, these data suggest that autophagy is a beneficial response which may help to limit MV-induced force loss in the diaphragm.

## Discussion

This study demonstrates that autophagy is acutely induced in the diaphragm as a specific response to MV. Therefore, our findings lead us to reject our initial hypothesis that MV would have a suppressive effect on autophagic flux in the diaphragm. Moreover, in this well-characterized mouse model of VIDD,<sup>4,8</sup> we demonstrate that autophagy activation in the diaphragm during MV is not causally implicated in its oxidative stress–induced force loss.<sup>4,5</sup> In fact, administration of the antioxidant NAC appeared to further promote autophagy in the diaphragm during MV while at the same time completely preventing VIDD. Furthermore, the classical autophagy-inducing compound rapamycin also helped to prevent MV-induced diaphragmatic force loss. Taken together, these data suggest that inhibition of autophagy is unlikely to be a fruitful avenue



**Fig. 6.** Antioxidant treatment does not suppress autophagy in the diaphragm during mechanical ventilation (MV). (A) Comparison of messenger RNA transcript levels (expressed as fold-change relative to average control [CTRL] value) for autophagy-related genes in MV and MV + *N*-acetylcysteine (NAC) mice. (B) Representative immunoblot showing LC3B-II levels in MV and MV + NAC mice, in either the absence or presence (+COL) of previous colchicine administration. (C) Quantification of autophagosome formation and degradation in these groups using the same analysis described in figure 4B. Autophagosome formation was increased in the MV + NAC group (mean, 3.5; 95% CI, 3.1 to 3.8) compared with the MV cohort (mean, 2.6; 95% CI, 2.1 to 3.0). No significant difference in autophagosome degradation was found between MV and MV + NAC groups. \**P* < 0.05 versus MV (unpaired *t* test, *n* = 8 mice per group). COL = colchicine.



**Fig. 7.** *N*-acetylcysteine (NAC) and rapamycin (RAPA) both prevent specific force loss in the diaphragm during mechanical ventilation (MV). Diaphragm strips were studied *ex vivo* to evaluate force-generating capacity at different frequencies of electrical stimulation. (A) Compared with the control (CTRL) group, MV diaphragms were significantly weaker at all stimulation frequencies. Pretreatment with NAC completely prevented the MV-induced force loss. \**P* < 0.05 for MV versus CTRL (two-way ANOVA at each stimulation frequency, *n* = 4 mice per group). (B) Compared with the CTRL group (same as A), the MV group (also treated with drug vehicle) demonstrated reduced force generation, whereas this was significantly prevented in the MV group treated with RAPA. \**P* < 0.05 for MV versus CTRL; †*P* < 0.05 for MV versus MV + RAPA (two-way ANOVA at each stimulation frequency, *n* = 6 mice per group).

for therapeutic intervention in VIDD. On the contrary, we speculate that autophagy pathway activation in the diaphragm may be a critical adaptive response to the

physiologic stress associated with MV, which might be usefully stimulated to an even greater degree as a potential treatment for VIDD.

The form of autophagy examined in this investigation, formally referred to as macroautophagy, is typified by the formation of double-membraned autophagosomes that can be identified by electron microscopy as well as biochemically by measuring lipidated LC3B-II protein levels. We found that MV increased the quantity of autophagosomes present in the diaphragm as ascertained by both of these methods. Importantly, we used colchicine to interfere with microtubule-dependent delivery of mature autophagosomes to lysosomes and thereby inhibit their degradation as previously described<sup>25,27,29</sup> to examine the influence of MV upon the balance between autophagosome formation and degradation. By using this approach in mice with appropriately controlled food intake, we found that MV independently and specifically led to an increase in autophagosome production by the diaphragm. Evidence for respiratory muscle target specificity of MV as a stimulus for autophagy was provided by the lack of MV-induced autophagy in the EDL, despite this limb muscle showing a markedly greater sensitivity to autophagy pathway activation after a prolonged period (48 h) of fasting.

Previous studies, one in human brain-dead organ donor patients<sup>7</sup> and others in rodents,<sup>17,18</sup> have reported an increase in LC3B-II protein levels in the diaphragm during MV. However, the interpretation of these findings was significantly hampered by experimental limitations, which prevented any definitive conclusions about a causal link between the use of MV and induction of autophagy in the diaphragm. For example, in the human study, most of the patients had been hospitalized for several days in the intensive care unit, and their nutritional status was not characterized.<sup>7</sup> Similarly, animals undergoing MV in previous studies were deprived of food intake for up to 18 h, which is a sufficient period of time to induce autophagy.<sup>29</sup> These previous studies were also unable to determine whether autophagosome accumulation in the diaphragm was related to increased autophagosome production or an impairment of autophagosome degradation (reduced "autophagic flux").

With respect to this last point, it is important to recognize that the number of autophagosomes present in a cell reflects a dynamic equilibrium between the rates of autophagosome formation and degradation. This dynamic balance is clearly illustrated in our study by the fact that the large increase in LC3B-II levels induced by 48 h of fasting in the EDL muscle was only evident when colchicine was used to prevent autophagosome degradation. Without the use of colchicine, the increased autophagosome production induced by prolonged fasting in the EDL would have been masked by equally accelerated rates of autophagosome degradation. These findings illustrate the utility of autophagosome degradation inhibitors such as colchicine for the interpretation of LC3B-II levels,<sup>19</sup> which has not been done in previous studies attempting to assess autophagy pathway activation in the diaphragm during MV.

Increased oxidative stress can stimulate autophagy<sup>24,25</sup> and is critically involved in MV-induced specific force loss of

the diaphragm.<sup>9–11</sup> It was also previously reported that inhibition of autophagy improved muscle morphology and locomotor function in a mouse model of congenital muscular dystrophy.<sup>31</sup> This raised the question of whether autophagy triggered by oxidative stress could be at least partly responsible for MV-induced weakness of the diaphragm. However, although antioxidant therapy with NAC completely prevented the development of MV-induced diaphragmatic weakness in our model, it did not suppress autophagy and was to the contrary associated with evidence for augmented autophagosome formation. This is consistent with the fact that an excess of reactive oxygen species can also inhibit autophagy<sup>32</sup> and may indicate a novel mechanism (*i.e.*, stimulation of autophagy) through which NAC exerts its beneficial effects upon VIDD.

The idea that stimulation of autophagy helps to mitigate VIDD is further supported by our results in mechanically ventilated mice treated with rapamycin, a standard and frequently used activator of the autophagy pathway.<sup>19</sup> Because rapamycin exerts its major biological effects by inhibiting mammalian target of rapamycin, a regulator of multiple intracellular processes, we cannot definitively conclude that its benefits for diaphragmatic function during MV in our study were solely related to stimulation of autophagy. We also cannot exclude the possibility that autophagy could play a harmful role in the eventual development of diaphragm atrophy during MV because the duration of MV was relatively short in our study and reflects the early stages of VIDD characterized mainly by a loss of specific force rather than fiber atrophy.<sup>4</sup> Nevertheless, our findings are in line with the fact that autophagy plays a key adaptive role in the cellular response to different forms of physiological and pathological stress.<sup>14,15</sup>

Indeed, autophagy appears to be crucial for maintaining normal skeletal muscle health. In this regard, Hermans *et al.*<sup>33</sup> reported an independent correlation between the activation of autophagy and improved muscle function in the critically ill. It has also been suggested that the increased vacuoles and LC3B-II levels found in the nonrespiratory muscles of critically ill patients may indicate impaired autophagy<sup>20</sup> although these findings are difficult to interpret without knowing the relative contributions of autophagosome production *versus* degradation as discussed earlier. Autophagy gene-deficient knockout mice demonstrate fiber atrophy as well as increased numbers of abnormal mitochondria in skeletal muscles,<sup>34,35</sup> and defective autophagy together with increased muscle fiber apoptosis is found in muscular dystrophy associated with collagen VI deficiency.<sup>36</sup>

Recent studies suggest that pathological oxidative stress arising from dysfunctional mitochondria plays a key role in VIDD pathogenesis.<sup>8,12,13</sup> Given the ability of autophagy to rid the cell of dysfunctional mitochondria (mitophagy), we speculate that autophagy is more likely to be an adaptive response to, rather than a cause of, the pathophysiological events leading to VIDD. In fact, the unique ability of autophagy to remove damaged organelles from cells raises the possibility

that one might be able to exploit this pathway to mitigate VIDD as has been shown recently in other muscle diseases. For instance, Pauly *et al.*<sup>37</sup> used an activator of AMP-activated protein kinase to induce autophagy in mdx mice (model of Duchenne muscular dystrophy) and observed a reduction in dysfunctional mitochondria exhibiting abnormal sensitivity to calcium-induced permeability transition pore opening, along with improved diaphragm muscle strength. Similarly, in mice with muscle disease due to collagen VI deficiency and defective autophagy, forced activation of the autophagy pathway by rapamycin or dietary approaches (starvation or low-protein diet) led to improvements of muscle structure and function.<sup>36</sup> Pharmacological stimulation of autophagy has also recently been reported to attenuate liver injury and improve survival in the cecal ligation-puncture model of sepsis in mice.<sup>21</sup> Therefore, it is conceivable that a strategy of stimulating autophagy might be useful in the prevention or treatment of VIDD although this remains to be determined.

In conclusion, this study demonstrates that during MV, autophagy is specifically induced in the diaphragm over the same time frame as the development of VIDD. However, our data collectively indicate that autophagy is unlikely to be a significant contributor to the development of VIDD. Rather, it is more probable that autophagy in the diaphragm represents a beneficial adaptive response to physiological stress during MV, suggesting the potential for therapeutic stimulation of autophagy to mitigate VIDD, and this possibility deserves further investigation.

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## Competing Interests

The authors declare no competing interests.

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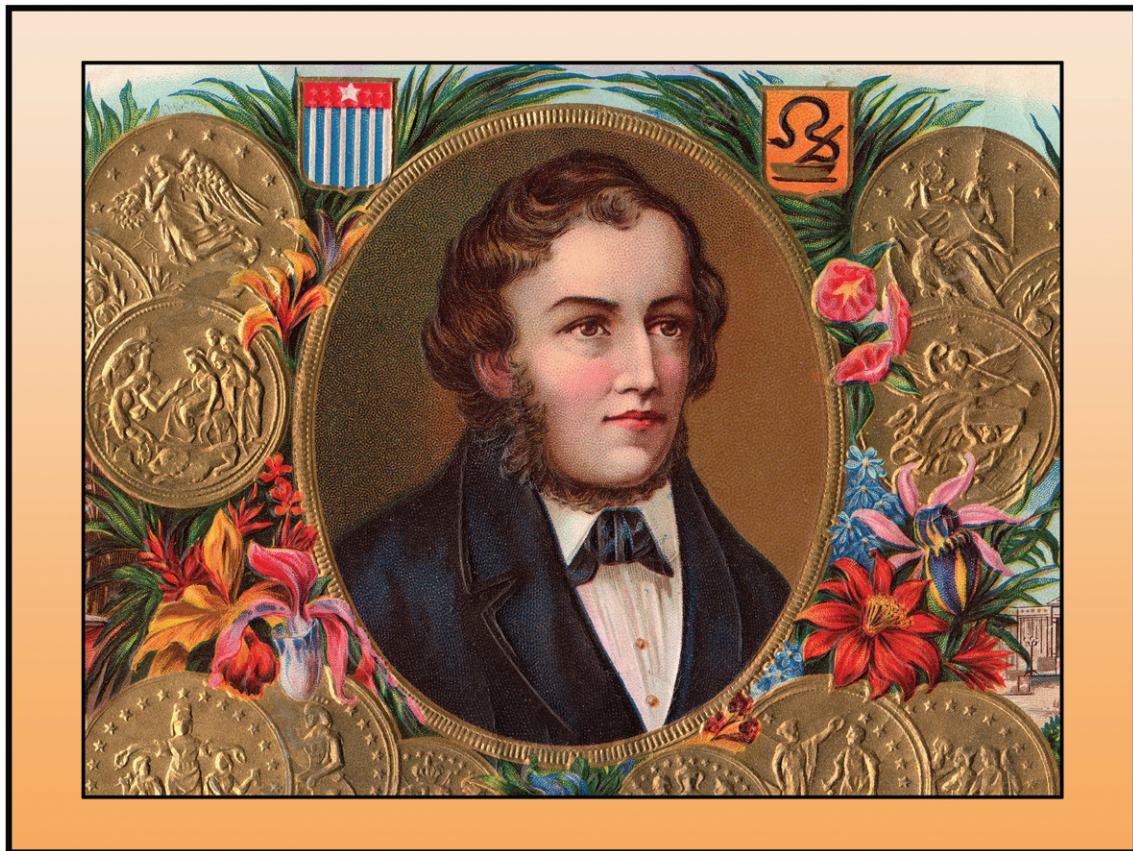
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## ANESTHESIOLOGY REFLECTIONS FROM THE WOOD LIBRARY-MUSEUM

### Identity Theft? “Tom Morton” Cigar Box Label, Part III



To the chagrin of today's anesthesiologists, from the 1880s to the 1910s, a certain “Tom Morton” was much better known to the American public than was ether pioneer “William T. G. Morton.” Tom Morton was a prolific English playwright whose wildly popular plays were featured on stages on both sides of the Atlantic. As one journalist noted, after a new cigar was branded “Tom Morton,” a “cigar manufacturer with the mind of a caterpillar and the morals of a Dick Turpin” [an English highwayman and horse thief] arbitrarily advertised the cigar not with the face of playwright Tom, but with the conveniently available visage of etherist William ... who was never known as “Tom Morton.” At least one portrait of playwright Tom depicts him as unbearded, unlike WTGM's bearded image (*above*), which is centered on the “Tom Morton” cigar box label. (Copyright © the American Society of Anesthesiologists, Inc.)

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