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Redox Balance and Cellular Inflammation in the Diaphragm, Limb Muscles, and Lungs of Mechanically Ventilated Rats

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ABSTRACT

Background: High tidal volume (V_T) mechanical ventilation was shown to induce organ injury other than lung injury and systemic inflammation in animal models of ventilator-induced lung injury. The authors aimed to explore whether high V_T mechanical ventilation *per se* induces early oxidative stress and inflammation in the diaphragm, limb muscles, and lungs of healthy rats exposed to ventilator-induced lung injury.

Methods: Protein carbonylation and nitration, antioxidants (immunoblotting), and inflammation (immunohistochemistry) were evaluated in the diaphragm, gastrocnemius, soleus, tibialis anterior, and lungs of mechanically ventilated healthy rats and in nonventilated control animals (n = 8/group) for 1 h, using two different strategies (moderate V_T [V_T = 9 ml/kg] and high V_T [V_T = 35 ml/kg]).

Results: The main findings are summarized as follows: compared with controls, (1) the diaphragms and gastrocnemius of high-V_T rats exhibited a decrease in reactive carbonyls, (2) the soleus and tibialis of high- and moderate-V_T rodents showed a reduction in reactive carbonyls and malondialdehyde-protein adducts, (3) the lungs of high-V_T rats exhibited a significant rise in malondialdehyde-protein adducts, (4) the soleus and tibialis of both high- and moderate-V_T rats showed a reduction in protein nitration, (5) the lungs of high- and moderate-V_T rats showed a reduction in antioxidant enzyme levels, but not in the muscles, and (6) the diaphragms and gastrocnemius of all groups exhibited very low inflammatory cell counts, whereas the lungs of high-V_T rats exhibited a significant increase in inflammatory infiltrates.

Conclusions: Although oxidative stress and inflammation increased in the lungs of rats exposed to high V_T, the diaphragm and limb muscles exhibited a decline in oxidative stress markers and very low levels of cellular inflammation.

What We Already Know about This Topic

- High tidal volume ventilation produces oxidative stress and inflammation in the lung, but whether it produces similar effects in other organs is less clear

What This Article Tells Us That Is New

- In normal rats, high tidal volume ventilation produced minimal or no oxidative stress and inflammation in the skeletal muscle and the diaphragm
- High tidal volume ventilation may not induce diaphragmatic dysfunction *via* oxidative stress and inflammation

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◆ This article is accompanied by an Editorial View. Please see: Musch G, Wiener-Kronish JP: Effect of ventilator-induced lung injury on skeletal muscle oxidative balance. ANESTHESIOLOGY 2010; 112:279–81.

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ACUTE lung injury (ALI) and acute respiratory distress syndrome (ARDS) are common conditions in intensive care units. Regardless of the severity of respiratory failure, the most common cause of death in patients with ARDS is shock and multiorgan failure.[1] Despite the lifesaving potential of-mechanical ventilation in patients with ALI and ARDS, complications arising out of this sort of therapy in critically ill patients should also be considered. In this regard, previous studies have shown that mechanical ventilation strategies using high tidal volume (V_T) and low positive end-expiratory pressure (PEEP) cause alveolar disruption and pulmonary edema, thus activating inflammatory pathways.[2,3] On this basis, it became clear that in patients with ARDS and ALI, certain strategies of mechanical ventilation might enhance lung injury, a condition known as ventilator-induced lung injury (VILI) .[4] The pathophysiology of VILI, however, has not yet been fully elucidated.

Oxidative stress, defined as the imbalance between oxidants and antioxidants in favor of the former, has been suggested to be involved, among other factors, in the development and perpetuation of VILI.[5] For instance, a reduction in antioxidant activity along with an increase in malondialdehyde was observed in the fluid lining of mechanically ventilated lungs.[6,7] In addition, protein tyrosine nitration, a marker of nitrosative stress, was also shown to be increased in the lungs of patients with ALI.[8] Interestingly, evidence from animal studies demonstrated that inflammatory cells and proinflammatory cytokines also contributed to the pathogenesis of VILI.[9,10]

Recently, the line has also been put forward that high V_T mechanical ventilation induced organ injury other than lung injury and systemic inflammation in several animal models of VILI.[11–17] It remains unknown, however, whether high V_T mechanical ventilation exerts deleterious effects on skeletal muscles, including the diaphragm, thus potentially influencing the weaning of patients from the ventilator. Moreover, studies investigating early molecular events induced by VILI in different organs and tissues are also lacking. On this basis, our study explored whether high V_T mechanical ventilation *per se* may induce early molecular changes such as increased oxidative stress levels and inflammation in the diaphragm, limb muscles, and lungs in an *in vivo* experimental model of VILI applied to healthy rats for 1 h. In fact, the VILI model used in the current investigation has already been used for exclusively exploring pulmonary and systemic effects of high V_T in healthy animals.[10–19] Accordingly, our objective was to specifically explore early molecular events involving oxidative stress, antioxidant mechanisms, and inflammation in the diaphragms, limb muscles (fast-and slow-twitch types), and lungs of healthy rats exposed to high V_T mechanical ventilation for 1 h.

Materials and Methods

Animals and Study Protocol

All animal experiments were conducted at the Hospital Universitario de Getafe (Madrid, Spain). This is a controlled study designed in accordance with both the ethical standards on animal experimentation (EU 609/86 CEE, Real Decreto 1201/05 BOE 252, Spain) at the Hospital Universitario de Getafe and the Helsinki convention for the use and care of animals. All experiments were approved by the Animal Research Committee at the Hospital Universitario de Getafe.

Three independent groups of pathogen-free healthy male Sprague-Dawley rats (Harlan Iberica, Spain, weight 325–375 g, n = 8/group) were studied using well-validated methodologies, which have been previously published by our group and others.[10–19] Two different ventilatory strategies were used in healthy rats to evaluate the effects of lung overdistension in the presence or absence of PEEP[10–19]: moderate V_T mechanical ventilation (V_T 9 ml/kg, PEEP = 5 cm H_2O), Group 1, and high V_T ventilation (V_T 35 ml/kg, PEEP = 0 cm H_2O), Group 2. In both groups of rats, respiratory parameters were set as follows: respiratory rate, 70 bpm; inspiratory time, 0.3 s; expiratory time, 0.56 s; and inspired fraction of oxygen, 0.45. All animals were initially ventilated for an equilibration period of 10 min using moderate V_T ventilation parameters before they were assigned to either Group 1 or Group 2 (moderate and high V_T mechanical ventilation, respectively). Both groups of animals were mechanically ventilated for 1 h, after being assigned to the corresponding group of the mechanical ventilation strategy. A third group of nonventilated rats (intact animals) was also studied (Group 3, control). Importantly, in ventilated animals, respiratory drive was abolished by the high respiratory rate set in the ventilator, because no spontaneous breaths were observed. Lung mechanics, hemodynamics, and circulatory conditions were measured both at time 0 and after the 1-h period of mechanical ventilation in each group of ventilated rats.

At the end of the experimental period (1 h), the following muscles and organs were removed in all animals during anesthesia: diaphragm, gastrocnemius, soleus, tibialis anterior, and lungs. The muscle and organ specimens were immediately frozen in liquid nitrogen and subsequently stored at −80°C. Furthermore, tissue specimens corresponding to the diaphragm, gastrocnemius, and lungs were also immersed in an alcohol–formol bath for 2 h and were embedded in paraffin. Frozen tissues were used for the redox marker analyses (immunoblotting), whereas paraffin-embedded tissues were used for the assessment of inflammation (immunohistochemical analyses). (See also Animals and Study Protocol in Supplemental Digital Content 1, in which full details on the animal experimentation and protocol is provided, http://links.lww.com/ALN/A569.)

Biologic Studies

All biologic analyses were conducted in the same laboratory at the IMIM-Hospital del Mar (Barcelona, Spain).

Oxidative Stress Markers. The effects of reactive oxygen species (ROS) and reactive nitrogen species on muscle proteins were evaluated using immunoblotting according to the methods published previously.[20–26] (See also Oxi-

Table 1. Changes in Blood Gases, Hemodynamic, and Mechanical Ventilatory Parameters in Rats Subjected to Mechanical Ventilation with Moderate-tidal (9 ml/kg) and High-tidal Volume (35 ml/kg)

	9 ml/kg (n = 8)		35 ml/kg (n = 8)	
	t = 0 min	t = 60 min	t = 0 min	t = 60 min
P_{aw} (cm H_2O)	15.3 ± 1.6	15.3 ± 1.9	41.1 ± 1.1	48.6 ± 2.7, $P < 0.001$
C_{RS} (ml/cm H_2O)	0.36 ± 0.05	0.33 ± 0.05	0.31 ± 0.02	0.23 ± 0.03, $P < 0.001$
MAP (mmHg)	121.9 ± 21.4	120.8 ± 16.6	134.0 ± 15.1	66.8 ± 14.5, $P < 0.001$
pH	7.30 ± 0.03	7.30 ± 0.04	7.30 ± 0.03	7.18 ± 0.05, $P < 0.001$
Lactate (mmol/L)	2.89 ± 0.46	2.59 ± 0.91	2.06 ± 0.64	4.03 ± 1.11, $P = 0.001$
Pao_2 (mmHg)	169.7 ± 13.0	175.9 ± 9.8	170.4 ± 7.0	62.8 ± 37.7, $P < 0.001$
$Paco_2$ (mmHg)	40.2 ± 10.3	41.0 ± 7.9	41.0 ± 9.7	45.4 ± 9.1, $P = 0.36$

Data are expressed as mean (SD). Variables are measured at baseline (t = 0 min) and after 1 h of mechanical ventilation (t = 60 min). C_{RS} = dynamic respiratory system compliance; MAP = mean arterial pressure; P_{aw} = peak airway pressure; $Paco_2$ = arterial partial pressure of carbon dioxide; Pao_2 = arterial partial pressure of oxygen.

dative Stress Markers in Supplemental Digital Content 1, in which full details on the redox balance analyses are provided, http://links.lww.com/ALN/A569.)

Inflammation in the Diaphragm and Gastrocnemius Muscles. To evaluate the presence of inflammatory cells in these muscles, immunohistochemical analyses were conducted in all the diaphragms and gastrocnemius of the three groups of animals by using similar methods published else where.[27,28] (See also Inflammation in Diaphragm and Gastrocnemius Muscles in Supplemental Digital Content 1, in which full details on the muscle inflammation assessment are described, http://links.lww.com/ALN/A569.)

Inflammation in the Lungs. Inflammatory infiltrates were determined in the lungs of the three groups of rats using hematoxylin-eosin staining according to the methodologies previously published.[29,30] See also Inflammation in the Lungs in Supplemental Digital Content 1, in which full details on the evaluation of lung inflammation levels are provided, http://links.lww.com/ALN/A569.

Statistical Analysis

This study is designed on the basis of a two-tailed hypothesis. Clinical and physiologic data are presented as mean (SD). Biologic data are presented as median and interquartile range in the tables, whereas they are presented as box and whisker plots in the figures. Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS, 12.0 version for windows, SPSS Inc., Chicago, IL). Mechanical ventilation-induced changes in clinical and physiologic variables were explored using paired Student-*t* test compared with baseline in both moderate- and high-V_T rat groups. Kruskal-Wallis test was used to examine significant differences among the three study groups, for each biologic variable and tissue. Furthermore, nonparametric Mann–Whitney U test was also used to specifically explore significant differences in biologic variables of the study between each group of mechanically ventilated rats and control animals. The sample size was based on previous studies[10–19] and on assumptions of 80% power to detect an improvement of more than 20% in measured outcomes at a level of significance of *P* less than or equal to 0.05. In all the biologic variables involving the study of redox balance (total reactive carbonyls, malondialdehyde-protein adducts, protein tyrosine nitration, Mn-superoxide dismutase (Mn-SOD), and catalase), mean difference between groups was estimated at a minimum of 25%, and SD was approximately 25–30% of the mean value for each of the variables.

Results

Effects of Mechanical Ventilation

Compared with time 0, peak airway pressure was significantly increased in rats exposed to high V_T for 1 h, while exhibiting a significant decrease in the dynamic compliance of the respiratory system (table 1). Furthermore, high-V_T rats showed hypotension, hypoxemia, acidosis, and hyperlactatemia after the 1-h ventilation period compared with levels at time 0 (table 1).

Oxidative Stress Markers

Total Reactive Carbonyls. Representative immunoblots corresponding to protein carbonylation in the diaphragm and gastrocnemius muscles of the rats are illustrated in figures 1A and B, respectively. In the diaphragms, total protein carbonylation was significantly lower in high-V_T animals than in either moderate-V_T rats or control animals (fig. 1C). In the gastrocnemius, protein carbonylation levels were significantly reduced in high-V_T rats compared with control animals (fig. 1D). High-V_T and moderate-V_T animals showed significantly lower levels of muscle protein carbonylation in the soleus and tibialis anterior compared with control rats (figs. 1E and F, respectively). In the rat lungs, reactive carbonyl levels did not significantly differ among the three study groups (table 2).

Malondialdehyde-protein Adducts. In the diaphragm and gastrocnemius of the rats (figs. 2A and B, respectively), total malondialdehyde-protein adducts levels did not significantly differ among the three groups. In the soleus and tibialis anterior (figs. 2C and D, respectively), high-V_T and moderate-V_T animals showed significantly lower levels of muscle

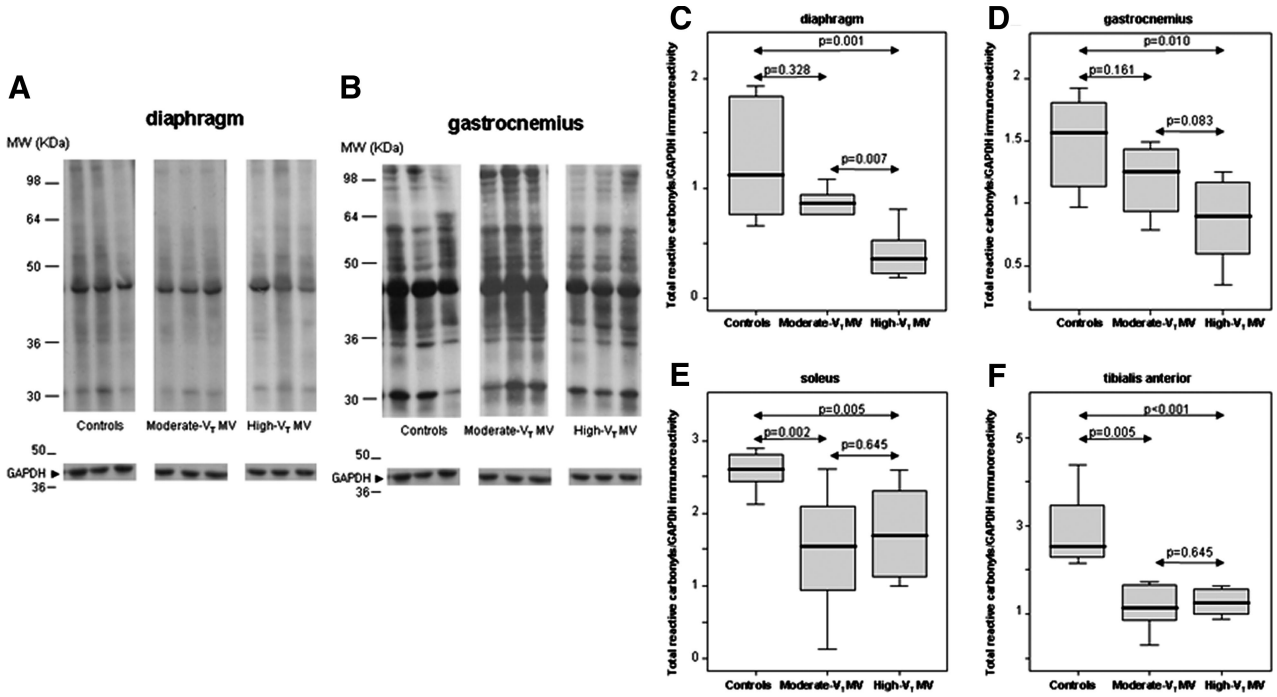

Fig. 1. Representative examples of protein oxidation (total carbonyl groups) immunoblots in the diaphragms (*A*) of control (n = 8), moderate tidal volume (V_T, n = 8), and high V_T (n = 8) mechanically ventilated rats. Several oxidized proteins of different molecular weights (MW, in kilo Daltons) were observed in all muscles. Representative immunoblots of oxidized proteins of different MW, in kilodaltons, in the gastrocnemius (*B*) of control, moderate-V_T, and high-V_T mechanically ventilated rats. Optical densities in the box plots are expressed as the ratio of the optical densities of total reactive carbonyl groups to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in each muscle. Standard box plots with median (twenty-fifth and seventy-fifth percentiles) and whiskers (at minimum and maximum values) are depicted. Reactive carbonyl levels were significantly reduced in the diaphragms (*C*) of the high-V_T rats compared with either control or moderate-V_T animals. In the gastrocnemius (*D*), reactive carbonyls were significantly decreased in high-V_T rats compared with controls and almost significantly reduced compared with moderate-V_T rodents. In the soleus (*E*) and tibialis anterior (*F*), reactive carbonyl levels were significantly decreased in high- and moderate-V_T rats compared with controls.

malondialdehyde-protein adducts compared with control rats. In the rat lungs, however, high-V_T animals showed significantly greater levels of total malondialdehyde-protein adducts compared with either moderate-V_T rats or control animals (figs. 2E and F).

Protein Tyrosine Nitra tion. In the diaphragm and gastroc-

Table 2. Oxidative Stress Markers in the Skeletal Muscles and Lungs of Mechanically Ventilated and Control Rats

	Controls (n = 8)	Moderate-V_T Mechanical Ventilation (n = 8)	High-V_T Mechanical Ventilation (n = 8)
Diaphragm and skeletal muscles			
Mn-SOD, a.u.			
Diaphragm	0.17 (0.13)	0.13 (0.12)	0.10 (0.13)
Gastronecmius	0.39 (0.25)	0.40 (0.11)	0.42 (0.36)
Soleus	0.89 (0.38)	0.58 (0.59)	0.81 (0.32)
Tibialis anterior	0.66 (0.20)	0.59 (0.05)	0.53 (0.15)
Catalase, a.u.			
Diaphragm	0.33 (0.12)	0.28 (0.07)	0.29 (0.08)
Gastronecmius	0.15 (0.06)	0.17 (0.09)	0.18 (0.05)
Soleus	0.69 (0.46)	0.62 (0.42)	0.65 (0.09)
Tibialis anterior	0.29 (0.40)	0.29 (0.13)	0.26 (0.22)
Lungs			
Total reactive carbonyls, a.u.			
Lungs	1.50 (0.35)	1.35 (0.59)	1.25 (0.26)
Protein tyrosine nitration, a.u.			
Lungs	0.43 (0.29)	0.47 (0.33)	0.31 (0.33)

Values are expressed as median (interquartile range).

a.u. = arbitrary units; Mn-SOD = manganese-superoxide dismutase; V_T = tidal volume.

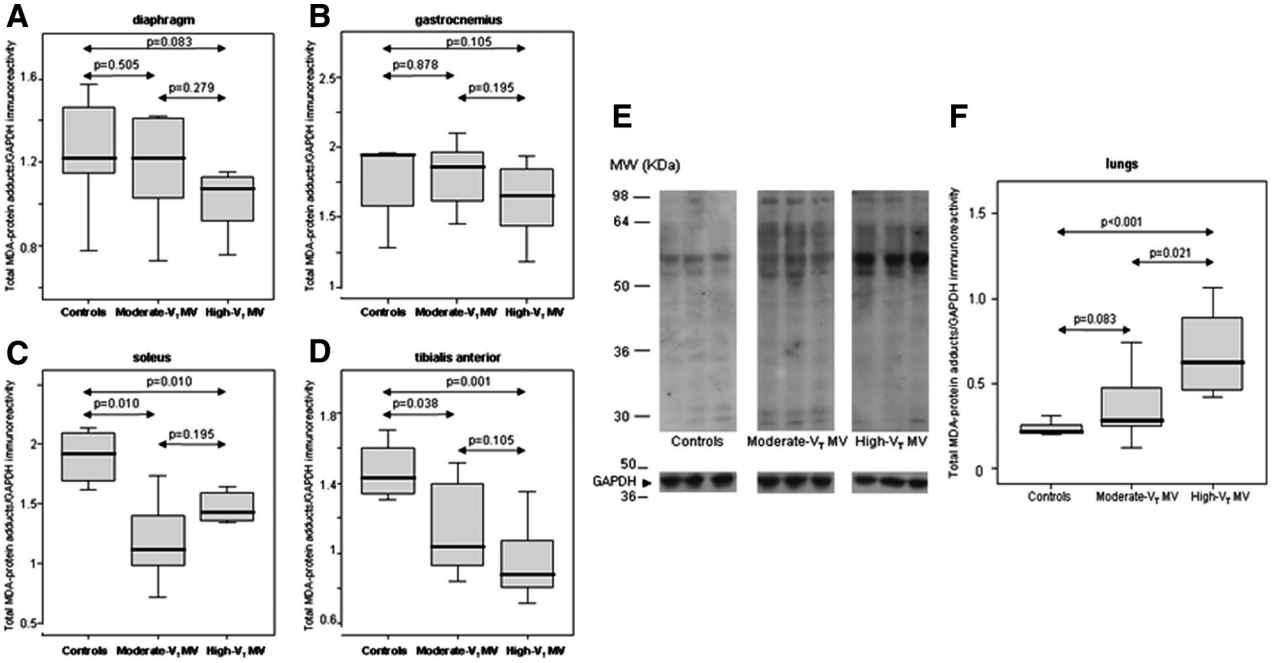

Fig. 2. Optical densities in the box plots are expressed as the ratio of the optical densities of total malondialdehyde (MDA)-protein adducts to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in each muscle. Standard box plots with median (twenty-fifth and seventy-fifth percentiles) and whiskers (at minimum and maximum values) are depicted. There was an almost significant decrease in MDA-protein adducts in the diaphragms (*A*) and gastrocnemius (*B*) muscles of the high-tidal volume (V_T, n = 8) rats compared with the controls (n = 8). In the soleus (*C*) and tibialis anterior (*D*), MDA-protein adduct levels were significantly decreased in high- and moderate-V_T rats (n = 8) compared with the controls. Representative immunoblots of MDA-protein adducts of different molecular weights (MW), in kilo Daltons, in the lungs (*E*) of control, moderate-V_T, and high-V_T mechanically ventilated rats. Optical densities in the box plots are expressed as the ratio of the optical densities of total MDA-protein adducts to those of GAPDH in the lungs (*F*). Standard box plots with median (twenty-fifth and seventy-fifth percentiles) and whiskers (at minimum and maximum values) are depicted (*F*). Lungs of high-V_T rats exhibited a significant increase in MDA-protein adducts compared with either controls or moderate-VT animals. The lungs of moderate-V_T rodents showed an almost significant increase in MDA-protein adducts compared with control animals (*F*).

nemius of the rats (figs. 3A and B, respectively), high-V_T rats showed significantly lower levels of muscle protein tyrosine nitration compared with moderate-V_T animals, but not controls. In the soleus and tibialis anterior of the rats (figs. 3C and D, respectively), high-V_T and moderate-V_T rats showed lower levels of muscle protein nitration compared with control animals. In the rat lungs, protein tyrosine nitration levels did not significantly differ among the three study groups (table 2).

Antioxidant Mechanisms

Mn-SOD. In the diaphragm, gastrocnemius, soleus, and tibialis anterior of the rats, Mn-SOD protein levels did not significantly differ among the three groups of animals (table 2). In the rat lungs, however, Mn-SOD protein levels were significantly reduced in both high-V_T and moderate-V_T animals compared with the controls (fig. 4A). Moreover, lung Mn-SOD levels were significantly lower in the high-VT rats compared with moderate-V_T animals (fig. 4A).

Catalase. In the diaphragm, gastrocnemius, soleus, and tibialis anterior of the rats, catalase protein levels did not significantly differ among the three groups of rats (table 2). Nevertheless, in the rat lungs, catalase protein levels were significantly reduced in both high-V_T and moderate-V_T animals compared with controls (fig. 4B).

Inflammatory Cells

Diaphragms and Gastrocnemius Muscles. Human tonsils were used as positive controls for both leukocyte and macrophage immunohistochemical identification (figs. 5A and B, respectively). Figures 5C and D illustrate inflammatory cell infiltration within the diaphragm fibers of a high-V_T rat and a control animal, respectively. Although intramuscular inflammatory cell infiltration was extremely low in the respiratory and limb muscles of the three study groups of rats, there was a statistically significant increase in inflammatory cell counts in the diaphragm and gastrocnemius (figs. 5E and F, respectively) of high-V_T rats compared with either controls or moderate-V_T animals.

Lungs. Hematoxylin-eosin staining of a lung section from both control and moderate-V_T rats are shown in figures 6A and B, respectively. Abundant inflammatory cells were observed within the lung inflammatory cell infiltrates in moderate-V_T rats (fig. 6C). Interestingly, lung inflammatory cell infiltrates were more abundant, but were of a smaller size, in high-V_T rats (figs. 6D and E) compared with moderate-V_T animals (fig. 6C). Abundant inflammatory cells were observed within the corresponding infiltrates (fig. 6F). The number of inflammatory infiltrates was significantly greater in the lungs of high-V_T rats compared with either control or moderate-V_T rodents (fig. 6G). However, total lung inflammatory infiltrated

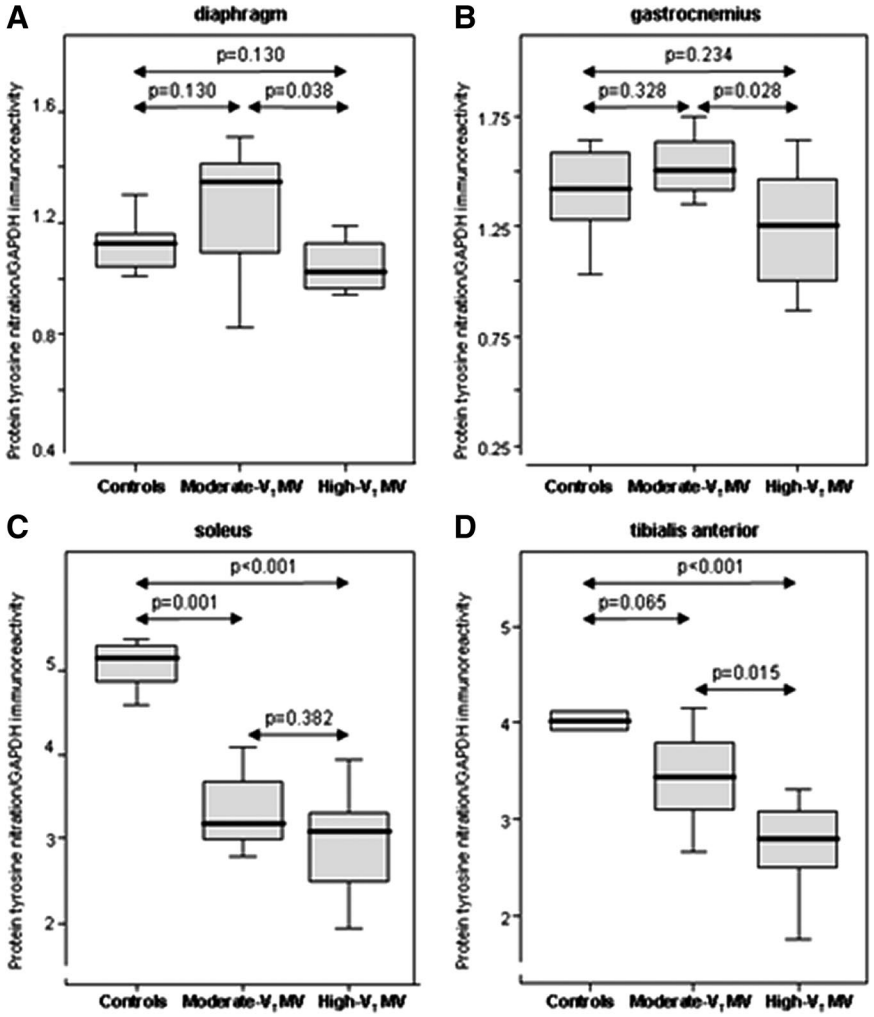

Fig. 3. Optical densities in the box plots are expressed as the ratio of the optical densities of total protein tyrosine nitration to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in each muscle. Standard box plots with median (twenty-fifth and seventy-fifth percentiles) and whiskers (at minimum and maximum values) are depicted. In the diaphragms (*A*) and gastrocnemius (*B*) muscles of the high-tidal volume (V_T, n = 8) mechanically ventilated (MV) rats, protein nitration levels were significantly reduced compared with moderate-V_T animals (n = 8) but not controls (n = 8). In the soleus (*C*) and tibialis anterior (*D*), protein tyrosine nitration levels were significantly decreased in high- and moderate-V_T rats compared with the controls.

area was significantly increased in both high- and moderate-V_T rats compared with control animals (fig. 6H).

Discussion

In this study, in contrast to our initial hypothesis, early molecular events after administration of high V_T mechanical ventilation to healthy rats were characterized by a significant reduction in both protein carbonylation and nitration in the diaphragm and limb muscles of the animals, while inducing a significant increase in protein oxidation in their lungs. Interestingly, in the muscle specimens, modifications in oxidative stress markers were not accompanied by any significant variation in the content of the antioxidant mechanisms Mn-SOD or catalase, whereas significant reductions in the levels of these antioxidant enzymes were observed in the lungs. Furthermore, although there was a statistically significant increase in inflammatory cell counts in the diaphragm and gastrocnemius muscles of high-V_T rats compared with control animals, such an increase was quite likely to be of small biologic relevance in terms of contribution to inflammatory and oxidative stress events in either respiratory or limb muscles of the mechanically ventilated animals. Finally, inflammatory cell infiltrates were greater in the lungs of mechanically ventilated rats compared with the nonventilated controls.

Muscle Redox Balance and Inflammation

In the current investigation, against our original hypothesis, high V_T mechanical ventilation induced a significant decrease in the levels of oxidative stress, as measured by protein carbonylation and nitration, in the diaphragms, gastrocnemius, soleus, and tibialis anterior muscles compared with nonventilated animals. Carbonyl formation (ketones and aldehydes) is an important detectable marker of protein oxidation. Carbonyl groups can be formed by the direct reaction of

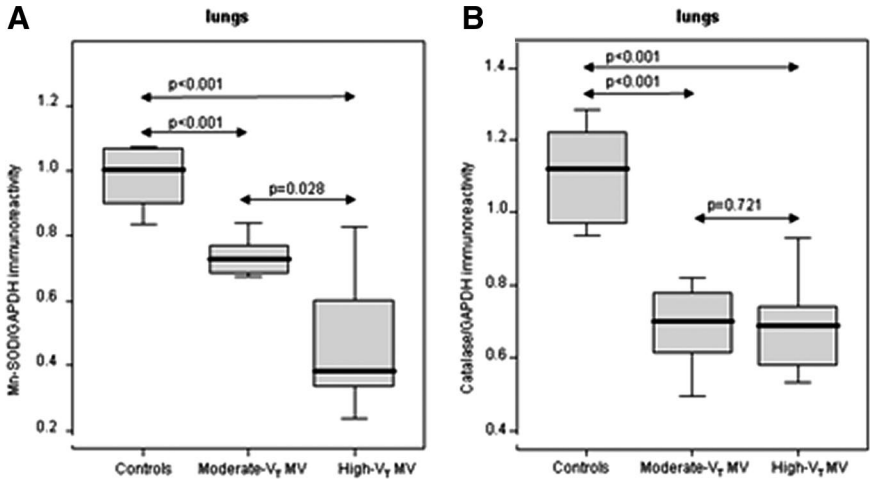

Fig. 4. Optical densities in the box plots are expressed as the ratio of the optical densities of Mn-SOD (*A*) and catalase (*B*) protein contents to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the lungs of the rat. Standard box plots with median (twenty-fifth and seventy-fifth percentiles) and whiskers (at minimum and maximum values) are depicted. Levels of Mn-SOD protein content (*A*) were lower in the lungs of high-tidal volume (V_T, n = 8) mechanically ventilated (MV) rats compared with either controls (n = 8) or moderate-V_T animals (n = 8). Levels of Mn-SOD content in the lungs of moderate-V_T rats were also significantly reduced compared with the controls. Levels of catalase protein (*B*) were lower in the lungs of high-V_T rats compared with controls, but not with those in moderate-V_T animals. Levels of catalase content in the lungs of moderate-V_T rats were also significantly reduced compared with the controls (*B*).

proteins with ROS, leading to the formation of protein derivatives containing highly reactive carbonyl groups,[31,32] and by Michael-addition reactions of lysine, cysteine, or histidine residues with unsaturated aldehydes (hydroxynonenal and malondialdehyde) formed during the peroxidation of polyunsaturated fatty acids.[31,32] Conversely, protein tyrosine nitration is usually considered a marker of excessive reactive nitrogen species production in the tissues. Peroxynitrite, which is formed from the near-diffusion limited reaction between nitric oxide and superoxide anions, is the most widely accepted mechanism of *in vivo* tyrosine nitration in the skeletal muscles.[33,34]

In the current investigation, modifications observed in protein carbonylation and nitration in both the respiratory and limb muscles of healthy animals ventilated at high V_T may also be part of the systemic effects of mechanical ventilation in the organs and systems other than the lungs. Indeed, high V_T mechanical ventilation of healthy rats was already shown to induce cardiovascular, liver, and gut injury in addition to systemic inflammation.[11–14,17,19] In this study,

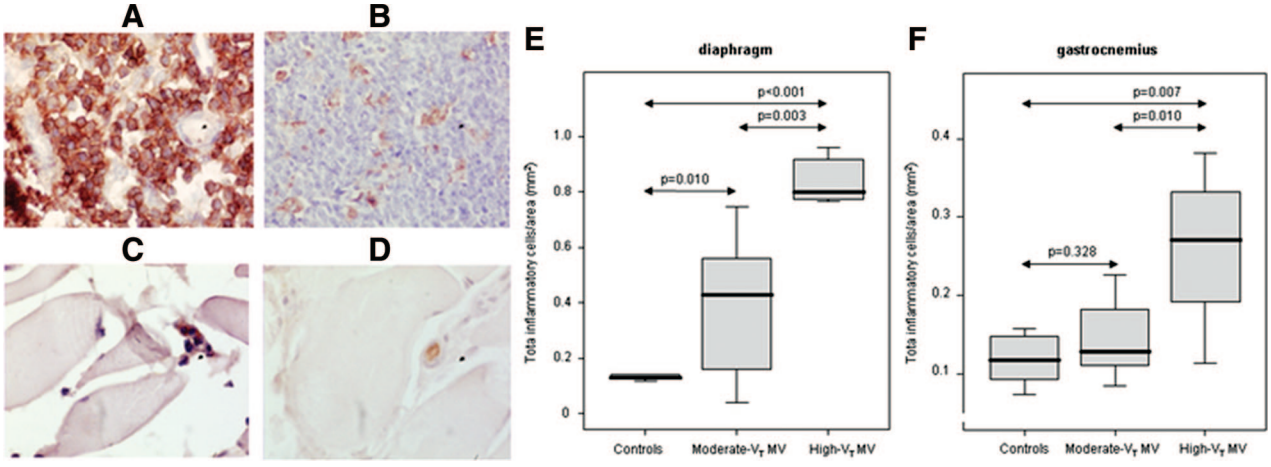

Fig. 5. Immunohistochemical localization of both leukocytes (*A*) and macrophages (*B*) in human tonsils (positive controls, ×400). Although intramuscular inflammatory cell levels were low in all groups of rats, diaphragm fibers of high-tidal volume (V_T) rats exhibited more prominent infiltration (*C*) than the control diaphragms (*D*). Standard box plots with median (twenty-fifth and seventy-fifth percentiles) and whiskers (at minimum and maximum values) are depicted. Total inflammatory cell counts (inflammatory cells/muscle section area in square millimeters [mm^2]) were significantly greater in the diaphragms (*E*) of high-V_T mechanically ventilated (MV) rats (n = 8) compared with either moderate-V_T animals (n = 8) or the controls (n = 8). Diaphragm inflammatory cell counts were also greater in moderate-V_T rats than in the controls (*E*). In the gastrocnemius (*F*), inflammatory cell counts (inflammatory cells/muscle section area in mm^2) of high-V_T rats were significantly increased compared with either controls or moderate-V_T rats. Gastrocnemius inflammatory cell counts did not significantly differ between moderate-V_T rats and controls (*F*).

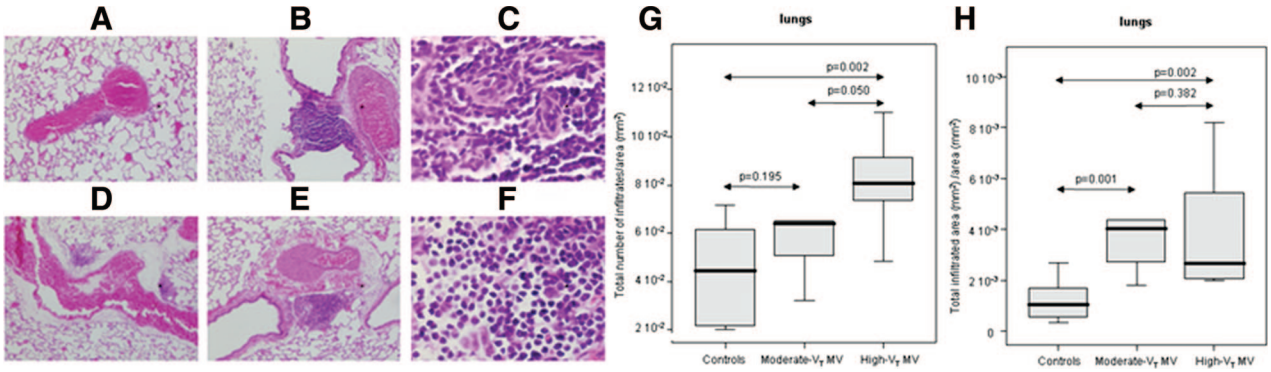

Fig. 6. Representative hematoxylin-eosin staining of a lung section from a control rat (*A,* ×40). Representative hematoxylin-eosin infiltrates corresponding to a lung section from a moderate tidal volume (V_T) rat (*B,* ×40). The presence of the abundant inflammatory cells in the same infiltrate can be seen at ×400 (*C*). High-V_T induced an increase in the number of lung inflammatory cell infiltrates (*D* and *E*, ×40). Abundant inflammatory cells were also seen in these infiltrates (*F,* ×400). Standard box plots with median (twenty-fifth and seventy-fifth percentiles) and whiskers (at minimum and maximum values) are depicted. The number of infiltrates (total number of infiltrates/total lung section area in square millimeters [mm^2]) was greater in the lungs of high-V_T mechanically ventilated (MV) rats (n = 8) compared with either control (n = 8) or moderate-V_T (n = 8) animals (*G*). The number of lung infiltrates did not differ between moderate-V_T rats and controls (*G*). Total lung inflammatory infiltrated area (total inflammatory infiltrated area in mm^2/total lung section area in mm^2) was significantly greater in high-V_T rats compared with controls, but not moderate-V_T rats (*H*). Total lung inflammatory infiltrated area was also significantly larger in the moderate-V_T rats compared with controls (*H*).

high-V_T ventilated rats exhibited hypotension, hypoxemia, acidosis, and hyperlactatemia—findings that are in keeping with previous reports.[17–19] Although the organ and skeletal muscle blood flow was not measured in this study, it would be possible to conclude that perfusion of organs and muscles in high-V_T rats was quite likely to be reduced as a result of the hypotension exhibited by these animals after the 1-h period of mechanical ventilation. Decreased blood flow and hypoxemia may well account for the reduced oxidative stress levels detected in both the respiratory and limb muscles of high-V_T rodents after mechanical ventilation compared with control animals. Interestingly, protein carbonylation (both reactive carbonyls and malondialdehyde-protein adducts) and nitration (3-nitrotyrosine immunoreactivity) levels were also significantly decreased in the soleus and tibialis anterior muscles of mechanically ventilated rats, using moderate V_T (9 ml/kg). Although not tested in the current investigation, it could be hypothesized that mechanical ventilation *per se* could alter vascular function and blood flow[15,19] in healthy animals, especially that of more distal and smaller muscles, such as the tibialis anterior and the soleus. Clearly, future studies will be designed to further explore this hypothesis.

It is worth noting that in none of the muscles examined, protein levels of the potent antioxidants Mn-SOD or catalase were modified by any of the mechanical ventilation strategies used in this study. These findings reinforce the concept that reductions in protein carbonylation and nitration levels detected in the diaphragm and limb muscles of mechanically ventilated healthy rats were most likely due to decreased ROS production within the skeletal muscle fibers. On the grounds that certain amounts of ROS and reactive nitrogen species are required for normal muscle force production, it could also be anticipated that decreased oxidative stress levels in the diaphragm and the limb muscles of mechanically ventilated animals may depress the muscle performance. Although not examined in the present investigation, Reid *et al.*[35] already demonstrated that depletion of ROS by incubation of diaphragm fiber bundles with the antioxidant catalase or by enzymatic removal of superoxide anions substantially depressed muscle contractile function. The results of their investigations led to the conclusion that in unfatigued muscles, ROS are mandatory for optimal contractile performance by facilitating, for instance, excitation-contraction coupling.[35] In conclusion, mechanical ventilation-induced oxidative stress cannot be assumed in all organs or tissues, at least in early stages. Administration of antioxidants[36–38] might not all be justified in these specific models of VILI.

Despite the statistically significant increase in inflammatory cells detected in the diaphragms of ventilated rats (both high and moderate V_T) and in the gastrocnemius of high-V_T animals, such results are likely to be of small biologic relevance, because total levels of inflammatory cells were, in fact, extremely low in both respiratory and limb muscles of the three groups of rodents. These findings are in keeping with recent data obtained in our laboratory (Esther Barreiro, M.D., Ph.D., unpublished observations, 2009), in which intramuscular inflammatory cell counts were also shown to be extremely low in the diaphragm, external intercostals, and vastus lateralis muscles of chronic obstructive pulmonary disease patients and control subjects.

Lung Redox Balance and Inflammation

A redox imbalance in the lungs has already been demonstrated in several models of VILI.[5–8] In our study, protein carbonylation levels, as measured by malondialdehyde-protein adducts, but not protein tyrosine nitration, were significantly greater in the lungs of high-V_T rats than in controls. Lung content of the antioxidants Mn-SOD and catalase, however, were significantly reduced in both groups of mechanically ventilated rodents compared with controls. Previ-

ous reports have also shown a decrease in antioxidant capacity in healthy lungs exposed to high V_T ventilation[6,39] and an increase in malondialdehyde concentrations in the alveolar lining fluid.[7] The underlying mechanisms accounting for such a redox imbalance are probably based on the cyclic stretch of the lungs induced by mechanical ventilation. In this regard, exposure of the lung epithelial and endothelial cells to cyclic stretch increased ROS production as early as 30 min.[40,41] Besides, intense cyclic stretch also reduces glutathione and increases glutathione oxidation,[40,42] suggesting that redox imbalance as a result of cyclic stretch is a major contributor to the onset and perpetuation of VILI.[5]

On the basis of the previous documented immunohistochemical tyrosine nitration of lung proteins in patients with ARDS,[8] nitrosative stress through protein nitration by reactive nitrogen species was also a proposed contributor to the pathogenesis of ALI. In this study, however, lung protein tyrosine nitration levels did not significantly differ among the three groups of animals. Moreover, we do not believe that tyrosine nitration of lung proteins through the action of leukocyte myeloperoxidases has taken place in mechanically ventilated rats, because the rise in inflammatory cell infiltrates was not accompanied by increased protein tyrosine nitration in their lungs. It is quite likely that mechanical ventilation for longer periods of time would have been associated with enhanced protein tyrosine nitration levels through either myeloperoxidase- or peroxynitrite-mediated mechanisms.

In the lungs of high-V_T ventilated rodents, the number and size of the inflammatory infiltrates was significantly greater than in the control group. Furthermore, the size of the infiltrated area, but not the number of infiltrates, was also significantly greater in moderate-V_T rats compared with nonventilated animals. This led us to the observation that inflammatory infiltrates, although less abundant, were slightly larger in moderate-V_T rats compared with high-V_T animals. Moreover, it should also be mentioned that the increase in inflammatory cell infiltrates observed in the lungs of mechanically ventilated animals is consistent with previous reports, in which the levels of several inflammatory cytokines, such as interleukin-1 β, interleukin-6, tumor necrosis factor-α, interleukin-8, and CXC chemokines,[10,43,44] were also shown to be increased in the lungs of healthy animals exposed to high V_T mechanical ventilation.

Study Critique

In the current investigation, the first limitation is related to the clinical relevance of the findings encountered by using this specific model of VILI, in which lung injury was induced by the administration of high V_T mechanical ventilation to healthy rats for a short period of time. In humans, 12 ml/kg has been shown to induce harmful effects on lungs,[45] whereas this V_T would be harmless in healthy rats. Furthermore, standard mechanical ventilation in humans has been recently shown to induce marked atrophy of the diaphragm fibers and increased proteolysis as a result of inactivity.[46] It is worth noting that the ventilatory strategies used in the current investigation were within the scope of those used in previously published reports from our group and other investigators,[10–19,47–49] in which key physiologic alterations defining the pulmonary and nonpulmonary effects of high V_T mechanical ventilation were demonstrated. In this regard, it is common practice to apply high-extreme conditions (clinical and/or physiologic) in completely healthy animals to generate contrast and to ensure that biologic differences among groups cannot be the subject of methodological concerns. On this basis, it is possible to conclude that compared with humans, administration of much larger V_T is required in healthy rats to elicit lung injury in addition to pathophysiologic and biologic responses. Furthermore, these different ventilatory strategies were used in this study with the aim of exploring lung overdistension in the presence or absence of PEEP. Importantly, in previously published investigations,[10–19] these ventilatory strategies were shown to yield similar end-inspiratory lung volumes. Hence, we believe that administration of a high V_T can be perfectly justified in this model.

The second limitation is related to the short period of mechanical ventilation administered to the animals. However, our main goal in this experimental study was to explore the early molecular events involving redox balance of slow- and fast-twitch muscles and lungs in response to high V_T mechanical ventilation in an animal model of VILI. Future studies will be definitely designed to assess medium- and long-term effects of VILI in organs and muscles by using a wider range of ventilatory strategies including lower V_T. Importantly, in the present investigation, inflammation and posttranslational oxidative modifications of both muscle and lung proteins have been evaluated in healthy rodents exposed to high V_T, thus showing significant variations in both the respiratory and limb muscles and the lungs compared with control animals.

Despite these recognized limitations, we believe that the study of events taking place very early after the pulmonary insult (high V_T stretch) is of clinical relevance, because early occurrence of oxidative stress during high V_T mechanical ventilation cannot be assumed in all organs or tissues. In fact, a reduction, rather than an increase, in oxidative stress was observed in both the respiratory and limb muscles of healthy rats exposed to large V_T and VILI for a relatively short period of time.

Conclusions

Our study provides evidence that even with the use of extremely large V_T and the creation of VILI in healthy rodents, there was no increase in oxidative stress in the diaphragm or limb muscles. In fact, at a particular time point, high V_T mechanical ventilation did not induce the same oxidative and inflammatory events in all the rat tissues, whereas oxidative stress and inflammation increased in the lungs and the diaphragm, and both slow- and fast-twitch limb muscles exhibited a significant decline in oxidative stress markers and low levels of intramuscular inflammatory cell infiltration.

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