Effects of elevated positive end-expiratory pressure on diaphragmatic blood flow and vascular resistance during mechanical ventilation

Andrew G. Horn,1 Dryden R. Baumfalk,1 Kiana M. Schulze,1 Olivia N. Kunkel,1 Trenton D. Colburn,1 Ramona E. Weber,1 Christian S. Bruells,2 Timothy I. Musch,1,3 David C. Poole,1,3 and Bradley J. Behnke1

1Department of Kinesiology, Kansas State University, Manhattan, Kansas; 2Department of Anesthesiology, Faculty of Medicine, RWTH Aachen University, Aachen, Germany; and 3Department of Anatomy and Physiology, Kansas State University, Manhattan, Kansas

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Horn AG, Baumfalk DR, Schulze KM, Kunkel ON, Colburn TD, Weber RE, Bruells CS, Musch TI, Poole DC, Behnke BJ. Effects of elevated positive end-expiratory pressure on diaphragmatic blood flow and vascular resistance during mechanical ventilation. J Appl Physiol 129: 626–635, 2020. First published July 30, 2020; doi:10.1152/japplphysiol.00320.2020.—Although mechanical ventilation (MV) is a life-saving intervention, prolonged MV can lead to deleterious effects on diaphragm function, including vascular incompetence and weaning failure. During MV, positive end-expiratory pressure (PEEP) is used to maintain small airway patency and mitigate alveolar damage. We tested the hypothesis that increased intrathoracic pressure with high levels of PEEP would increase diaphragm vascular resistance and decrease perfusion. Female Sprague-Dawley rats (~6 mo) were randomly divided into two groups receiving low PEEP (1 cmH2O; n = 10) or high PEEP (9 cmH2O; n = 9) during MV. Blood flow, via fluorescent microspheres, was determined during spontaneous breathing (SB), low-PEEP MV, high-PEEP MV, low-PEEP MV + surgical laparotomy (LAP), and high-PEEP MV + pneumothorax (PTX). Compared with SB, both low-PEEP MV and high-PEEP MV increased total diaphragm and medial costal vascular resistance (P ≤ 0.05) and reduced total and medial costal diaphragm blood flow (P ≤ 0.05). Also, during MV, medial costal diaphragm vascular resistance was greater and blood flow lower with high-PEEP MV vs. low-PEEP MV (P ≤ 0.05). Diaphragm perfusion with high-PEEP MV + PTX and low-PEEP MV were not different (P > 0.05). The reduced total and medial costal diaphragmatic blood flow with low-PEEP MV appears to be independent of intrathoracic pressure changes and is attributed to increased vascular resistance and diaphragm quiescence. Mechanical compression of the diaphragm vasculature may play a role in the lower diaphragmatic blood flow at higher levels of PEEP. These reductions in blood flow to the quiescent diaphragm during MV could predispose critically ill patients to weaning complications.

NEW & NOTEWORTHY This is the first study, to our knowledge, demonstrating that mechanical ventilation, with low and high positive-end expiratory pressure (PEEP), increases vascular resistance and reduces total and regional diaphragm perfusion. The rapid reduction in diaphragm perfusion and increased vascular resistance may initiate a cascade of events that predispose the diaphragm to vascular and thus contractile dysfunction with prolonged mechanical ventilation.

medial costal diaphragm; vascular function; ventilatory muscle failure

INTRODUCTION

Mechanical ventilation (MV) is a life-saving intervention employed to maintain adequate levels of pulmonary gas exchange. Importantly, with the current coronavirus disease 2019 (COVID-19) pandemic, the most common reason for COVID-19 patient ICU admission is severe hypoxic respiratory failure requiring MV (45). Prolonged MV in humans (>18 h) and in preclinical animal models (≥6 h) (11, 20, 30, 34) induces diaphragmatic atrophy and contractile dysfunction as well as oxidative stress and mitochondrial dysregulation, collectively referred to as ventilator-induced diaphragmatic dysfunction (VIDD) (44). In addition, the failure to wean patients effectively from ventilators increases patient morbidity and mortality (27). Although the etiology of VIDD is multifaceted, a primary mechanism that has received relatively scant attention is the O2 delivery (Q˙O2) to O2 utilization (V˙O2) mismatching within the diaphragm during prolonged MV (7).

Diaphragmatic blood flow is reduced with acute MV (i.e., measured ~30 min after induction of MV) (7, 15, 33, 43), as expected with the unloading of this muscle. However, prolonged inactivity of the diaphragm (i.e., 6 h of MV) results in further time-dependent lowering of perfusion and an inability to augment diaphragm blood flow with contractions (7). Therefore, the compromised diaphragm perfusion with prolonged MV would hasten fatigue (i.e., QO2 limitation constraining increases in VO2) and contribute to weaning difficulties. The precise mechanisms (s) for the time-dependent reductions in diaphragm perfusion with inactivity, which is not present in other highly oxidative muscles (7), is currently unknown. Importantly, prolonged MV induces diaphragm resistance vessel dysfunction (13) and elevated levels of hypoxia-inducible factor 1α (HIF-1α) (4), indicating rapid deficits in vasomotor control and tissue hypoxia, respectively.

During MV, patients may receive varying levels of positive end-expiratory airway pressure (PEEP) to maintain small airway patency and mitigate alveolar damage. However, the optimal level of PEEP has not been established and may be patient/condition dependent (8, 10). The use of PEEP can increase intrathoracic pressure and lung volume, thereby caudally displacing the diaphragm and reducing diaphragm sarcomere length (21, 32). Recently, the reduced diaphragm perfusion with positive-pressure MV has been attributed to caudal diaphragm displacement and supraphysiological intrathoracic pressure generation (42). Additionally, caudal diaphragm dis-
placement may increase intra-abdominal pressure and partially reduce diaphragm perfusion pressure. However, in dogs hyperinflation of the lungs does not alter diaphragmatic blood flow (18), suggesting minimal effect of caudal displacement on diaphragm perfusion. Currently, the effects of altered intra-abdominal and/or intrathoracic pressure on diaphragm perfusion with MV are unknown.

Therefore, the overall objective of this study was to investigate the effects of PEEP-induced intrathoracic and intra-abdominal pressure changes during MV on diaphragmatic blood flow in an established preclinical animal model. Specifically, we tested the following hypotheses during MV in the rat: 1) there will be an increase in diaphragm vascular resistance and a reduction in diaphragmatic blood flow at both 1 cmH2O and 9 cmH2O of PEEP compared with spontaneous breathing (SB), 2) ablation of intrathoracic pressures [via pneumothorax (PTX)] will increase diaphragmatic blood flow, and 3) surgical laparotomy (i.e., ablating intra-abdominal pressure) will not alter diaphragmatic blood flow. Given that PEEP is a necessity in many critically ill patients during MV, there is evidence that higher PEEP may be detrimental to patients (27).

These studies are clinically relevant to understanding the impact of PEEP on diaphragmatic blood flow during MV. Specifically, these studies may provide mechanistic insights into the bases for vasomotor dysfunction with prolonged MV (13). It is hoped these insights will help provide a foundation for designing targeted therapeutic strategies to augment diaphragmatic blood flow, manage critically ill patients, and facilitate successful weaning.

**METHODS**

**Animals.** Female Sprague-Dawley rats (n = 19, 4–8 mo old, ~335 g) obtained from Charles River Laboratories (Boston, MA) were subjected to spontaneous breathing in each experiment and were then randomly divided into two experimental groups: 1) low-PEEP mechanical ventilation (n = 10; Fig. 1A) and 2) high-PEEP mechanical ventilation (n = 9; Fig. 1A). Here, low PEEP is defined as 1 cmH2O and high PEEP is defined as 9 cmH2O. Our rationale for this low-versus high-PEEP classification comes from previously published literature focusing on VIDD in the rat and different levels of PEEP (35). The female Sprague-Dawley rat was chosen because of the similar properties (e.g., anatomical and physiological) of the rat diaphragm to the human diaphragm (i.e., fiber type composition, shape) (24, 25, 28), and this age range, healthy status, and the slower growth rate of females allow for experimental investigation in the absence of underlying pathologies and body mass differences among groups. In addition, VIDD occurs equally in males and females (20), and it is acknowledged that VIDD can be studied in both male and female rats. All procedures were approved by the Kansas State University Institutional Animal Care and Use Committee and complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Upon arrival, animals were housed and maintained in a temperature-controlled (23 ± 2°C) room with a 12:12-h light-dark cycle, with water and rat chow provided ad libitum.

**Surgical preparation.** All surgical procedures were performed with aseptic techniques. Rats were initially anesthetized with a 5% isoflurane-O2 mixture (isoflurane vaporizer; Harvard Apparatus, Cambridge, MA) and subsequently maintained on 3% isoflurane-O2. Body temperature was maintained at 37 ± 1°C (via rectal thermometer) by use of a water-recirculating heating blanket. An incision was made on the ventral side of the neck, and the left carotid artery was isolated and cannulated with PE-10 connected to PE-50 (Intra-Medic polyethylene tubing, Clay Adams Brand; Becton, Dickinson, Sparks, MD) for measurements of mean arterial pressure (MAP) (Digi-Med BPA; Micro-Med, Louisville, KY) and infusion of fluorescent microspheres (see below). A second catheter (PE-10 connected to PE-50) was inserted into the caudal artery for the infusion of pentobarbital sodium anesthesia and reference sampling for blood flow determination. Rats were then transitioned to pentobarbital sodium anesthesia (20 mg/kg body wt) given intra-arterially while concentrations of isoflurane were decreased and subsequently discontinued. The level of anesthesia was regularly monitored via toe pinch and palpebral reflex, with pentobarbital anesthesia supplemented (3.5–7.0 mg/kg) as necessary.

**Mechanical ventilation.** Rats were tracheostomized and connected to a volume-cycled rodent ventilator (Kent Scientific PhysioSuite, Fig. 1. The random distribution of animals after spontaneous breathing (SB; A) and the experimental paradigm (B). MV, mechanical ventilation; LAP, surgical laparotomy maneuver; PEEP, positive end-expiratory pressure; PTX, pneumothorax induction; Q, injection of microspheres for blood flow determination. Q1, Q2, and Q3 represent the 3 time points for fluorescent microsphere injection.

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Low-PEEP MV and low-PEEP MV + surgical laparotomy. Each animal \( n = 10 \) was mechanically ventilated with low PEEP (1 cmH\(_2\)O), and fluorescent microspheres were infused at three different time points as outlined in Fig. 1B. After the second fluorescent microsphere infusion (Fig. 1B), a surgical laparotomy (LAP) was performed on each animal to investigate the effects of intra-abdominal pressure changes on diaphragmatic blood flow. The xiphoid process was gently lifted upward with forceps to avoid damage to vital organs, and a small incision was made at the midline, directly below the xiphoid process. Then, the laparotomy was performed by cutting the abdominal wall (~2 cm) laterally from the midline on both sides of the animal. Thereafter, the third and final microsphere infusion was performed (Fig. 1B).

High-PEEP MV and high-PEEP MV + pneumothorax. Each animal \( n = 9 \) was mechanically ventilated with high PEEP (9 cmH\(_2\)O), and fluorescent microspheres were infused at three different time points (Fig. 1B). To assess the effects of intrathoracic pressure changes on diaphragmatic blood flow, animals were then subjected to a bilateral pneumothorax (PTX). After the second fluorescent microsphere infusion (Fig. 1B), the skin and superficial muscle tissue were removed to expose the outer surface of the ribcage and intercostal muscles. Then, a bilateral PTX was induced via a single thoracotomy incision at the right and left 4th intercostal space, followed by the third and final microsphere infusion (Fig. 1B).

Fluorescent microsphere injection. The fluorescent microsphere technique, as previously described (9, 26), was used to quantify tissue blood flow in each experimental group. As mentioned above, fluorescent microspheres were infused at three different time points: 1) during spontaneous breathing (SB), 2) 10 min after intubation and a stable PET\(_{\text{CO}}\), of ≤30 mmHg, and 3) 10 min after each surgical maneuver (LAP or PTX) (Fig. 1B). For each measure, a reference blood sample was taken from the tail artery catheter with a Harvard withdrawal pump (model 907; Cambridge, MA) that was initiated 30 s before microsphere infusion at a withdrawal rate of 0.25 mL/min and 2.0–2.5 \( \times 10^5 \) fluorescent microspheres (colors: red, scarlet, blue-green, 15.5-μm diameter; Invitrogen FluosphereS; Carlsbad, CA) were infused into the aortic arch via the carotid artery catheter. Adequate mixing of microspheres was determined by ≤20% difference in left and right kidneys or left and right soleus muscle blood flows at each time point. After the final microsphere infusion, while under a deep plane of anesthesia, rats were euthanized with pentobarbital sodium overdose (>50 mg/kg ia). Thereafter, tissues (diaphragm, soleus, external and internal intercostal muscles, and kidneys) were harvested, weighed, and placed in 15-mL screw-cap polypropylene conical tubes and then placed in a −80°C freezer for subsequent blood flow analysis. The diaphragm was sectioned into costal (ventral, medial, and dorsal) and crural portions to determine regional distribution of diaphragmatic blood flow, with the sum of these portions used to calculate total diaphragm blood flow.

Calculation of vascular resistance and blood flow. The fluorescent microsphere assay was performed according to Deveci and Egginton (9). Chemically digested tissue samples were placed into a 96-well plate, with each sample analyzed in quadruplicate. After the fluorescence intensity of each tissue and reference blood sample was measured with a Spectramax i3 plate reader (Molecular Devices, Sunnyvale, CA), vascular resistance was calculated as follows (9):

\[
VR = \frac{MAP}{Q}
\]

where VR is vascular resistance (mmHg/mL/min/100 g), \( Q \) is blood flow (mL·min\(^{-1}\)·100 g\(^{-1}\)), and MAP is the mean arterial pressure (mmHg) recorded immediately before microsphere infusion. Tissue blood flow was calculated as

\[
Q = \left( \frac{A_i/A_s}{s/w} \right) \times 100
\]

where \( Q \) is blood flow (mL·min\(^{-1}\)·100 g\(^{-1}\)), \( A_i \) is the individual sample intensity, \( A_s \) is the reference blood sample intensity, \( s \) is the withdrawal rate (0.25 mL/min) of the reference blood sample, and \( w \) is the tissue weight (g).

Data analysis. Data were analyzed with GraphPad Prism8 (GraphPad Software, San Diego, CA). Body mass (g), diaphragm mass (g), MAP (mmHg), HR (beats/min), and Sa\(_{\text{O}}\) (%) were analyzed with a one-way ANOVA (Table 1). In each experiment (Fig. 1A), tissue blood flows and vascular resistance during SB, low-PEEP MV, high-PEEP MV, surgical laparotomy, and pneumothorax were analyzed with a mixed-effects model. A Grubb’s outlier test was performed on all data, and a total of 19 outliers were removed before analysis, with only 1 outlier (i.e., the most extreme) removed from each respective data subset (e.g., the most extreme value of ventral costal blood flow from the spontaneous breathing condition in the high-PEEP MV group). Normal distribution was confirmed in the data set with a Shapiro–Wilk test, and homoscedasticity was assessed with a Bartlett’s test. Post hoc analyses were performed with a Holm–Sidak test. All data are presented as means ± SE, and statistical significance was established at \( P \leq 0.05 \). Of the 19 animals used for this investigation, poor microsphere mixing (described above) was present in 3 animals during low-PEEP MV + pneumothorax (\( n = 7 \)) and 3 animals during high-PEEP MV + pneumothorax (\( n = 6 \)).
RESULTS

Low-PEEP MV. Compared with spontaneous breathing (SB), total diaphragm vascular resistance was increased and total diaphragm blood flow diminished during low-PEEP MV \( (P \leq 0.05; \text{Fig. 2, A and C}) \). During low-PEEP MV, medial costal diaphragm vascular resistance was increased and there was a reduced medial costal diaphragm blood flow versus SB \( (P \leq 0.05; \text{Fig. 3, A and C}) \). Dorsal costal diaphragm vascular resistance was increased during low-PEEP MV versus SB \( (P \leq 0.05; \text{Table 2}) \). Soleus vascular resistance decreased and soleus blood flow increased during low-PEEP MV compared with SB \( (P \leq 0.05; \text{Table 2}) \). Intercostal vascular resistance and muscle blood flow were unchanged with low-PEEP MV \( (P \leq 0.05; \text{Table 2}) \). Total kidney vascular resistance was significantly increased during low-PEEP MV, with a concomitant decrease in total kidney blood flow \( (P \leq 0.05; \text{Table 2}) \).

High-PEEP MV. Total diaphragm vascular resistance was increased and total diaphragm blood flow was lower during high-PEEP MV versus SB \( (P \leq 0.05; \text{Fig. 2, B and D}) \). Medial costal diaphragm vascular resistance was higher and medial costal diaphragm blood flow was lower with high-PEEP MV compared with SB \( (P \leq 0.05; \text{Fig. 3, B and D}) \). Compared with SB, ventral and dorsal costal and crural diaphragm vascular resistances were higher with high-PEEP MV \( (P \leq 0.05; \text{Table 2}) \). Ventral and dorsal costal diaphragm blood flows were reduced with high-PEEP MV \( (P \leq 0.05; \text{Table 2}) \). Neither soleus nor internal or external intercostal muscles elicited any change in blood flow from SB to high-PEEP MV \( (P > 0.05; \text{Table 2}) \). Soleus vascular resistance increased during high-PEEP MV \( (P \leq 0.05; \text{Table 2}) \). Total kidney vascular resistance was increased and total kidney blood flow was reduced with high-PEEP MV versus SB \( (P \leq 0.05; \text{Table 2}) \).

Low-PEEP vs. high-PEEP MV. Total diaphragm vascular resistance was increased to a greater extent with high-PEEP MV versus low-PEEP MV \( (P \leq 0.05; \text{Fig. 4, A}) \). Consequently, high-PEEP MV elicited a larger reduction in total diaphragm blood flow versus low-PEEP MV \( (P \leq 0.05; \text{Fig. 4, C}) \). During high-PEEP MV, vascular resistance was higher and blood flow was lower in the medial costal diaphragm versus low-PEEP MV \( (P \leq 0.05; \text{Fig. 4, B and D}) \).

Surgical laparotomy and pneumothorax. During low-PEEP MV + LAP and high-PEEP MV + PTX, total diaphragm vascular resistance was lower versus high-PEEP MV alone \( (P \leq 0.05; \text{Fig. 4, A}) \) but not different compared with low-PEEP MV alone. 

Fig. 2. Total diaphragm vascular resistance and blood flow during spontaneous breathing \( (n = 10) \), low-positive end-expiratory pressure (PEEP) mechanical ventilation (MV) \( (n = 10) \), and low-PEEP MV + surgical laparotomy (LAP) \( (n = 7) \) (A and C) and total diaphragm vascular resistance and blood flow during spontaneous breathing \( (n = 9) \), high-PEEP MV \( (n = 9) \), and high-PEEP MV + pneumothorax (PTX) \( (n = 6) \) (B and D). *Significant \( (P \leq 0.05) \) difference vs. spontaneous breathing; †significant \( (P \leq 0.05) \) difference vs. high-PEEP MV.
MV alone ($P > 0.05$; Fig. 4A). Total diaphragm blood flow was increased with low-PEEP MV + LAP and high-PEEP MV + PTX compared with high-PEEP MV ($P \leq 0.05$; Fig. 4C) but not different versus the low-PEEP condition ($P > 0.05$; Fig. 4C). Within the medial costal diaphragm during low-PEEP MV + LAP and high-PEEP MV + PTX, vascular resistance was decreased and blood flow was higher versus high-PEEP MV ($P \leq 0.05$; Fig. 4B). There were no differences in medial costal vascular resistance or blood flow during low-PEEP MV + LAP and high-PEEP MV + PTX compared with low-PEEP MV ($P > 0.05$; Fig. 4, B and D).

**Heart rate, mean arterial pressure, and renal perfusion with MV.** Heart rate (HR) was increased during low-PEEP MV versus the SB condition ($P \leq 0.05$), and after surgical laparotomy HR returned to a value similar to that seen during SB (Table 1). In the high-PEEP MV group there were no differences in HR ($P > 0.05$; Table 1). Compared with SB, both low- and high-PEEP MV increased MAP, increased renal vascular resistance, and reduced renal blood flow ($P \leq 0.05$; Table 1). After surgical laparotomy, MAP was lower versus low-PEEP MV and higher versus SB ($P \leq 0.05$; Table 1). MAP with high-PEEP MV + PTX was lower versus high-PEEP MV ($P \leq 0.05$; Table 1). Renal vascular resistance was lower during both low-PEEP MV + LAP and high-PEEP MV + PTX, and renal perfusion increased in both conditions ($P \leq 0.05$; Table 2). Vascular conductance (mL/min/100 g/mmHg) was calculated for all tissues in each experimental condition (data not shown) and yielded results reciprocal to the vascular resistance data presented here.

**DISCUSSION**

Previously, the effects of different levels of PEEP on diaphragm perfusion were unknown. The present study demonstrates that both low- and high-PEEP MV augment total and medial costal diaphragm vascular resistance. The increased vascular resistance, coupled with diaphragm inactivity during MV, reduced bulk diaphragmatic and medial costal blood flow.
Furthermore, the reductions in diaphragmatic blood flow during acute MV with PEEP, similar to prolonged MV with zero PEEP (7), are specific to the diaphragm muscle, as blood flows to other respiratory and hindlimb muscles were not significantly reduced (Table 2). In addition, with increasing PEEP, diaphragm perfusion is diminished to a greater extent. The significantly reduced (Table 2). In addition, with increasing PEEP, diaphragm vascular conductance previously demonstrated with MV (7), low- and high-PEEP MV increased diaphragm vascular resistance, which resulted in the reduced diaphragm perfusion (Fig. 2, A and B). Importantly, the elevated vascular resistance and rapid blood flow reductions (i.e., ≤10 min) suggest an increased tonic vasoconstriction of the diaphragm vasculature during MV that is not seen in other respiratory and skeletal muscles (Table 2). Interestingly, high-PEEP MV increased vascular resistance and reduced blood flow in the whole diaphragm to a greater extent than low-PEEP MV (Fig. 4, A and C). The larger reduction in total diaphragm blood flow with higher PEEP may be related to a decrease in transmural pressure in the diaphragm vasculature during MV. This effect may arise from greater intrathoracic pressures and/or compressive effects on the thoracic aorta and possibly the phrenic artery, raising diaphragm arteriolar intravascular pressure and instigating myogenic vasoconstriction.

During low-PEEP MV, the ablation of intra-abdominal pressure (i.e., laparotomy) had no effect on total diaphragm vascular resistance or blood flow (Fig. 2, A and C). This suggests that low-PEEP MV, acutely, may not result in caudal diaphragm displacement. However, in the case of high-PEEP MV, total diaphragmatic vascular resistance compared to low-PEEP MV with laparotomy was significantly higher (Fig. 4A) and blood flow lower (Fig. 4C), suggesting a possible role of abdominal compression-mediated reductions in diaphragmatic blood flow with higher levels of PEEP. During high-PEEP MV, total diaphragm vascular resistance was lower with a pneumothorax versus high-PEEP MV alone but was not different versus low-PEEP MV (Fig. 4A). Diaphragmatic blood flow increased with a pneumothorax; however, the increased diaphragmatic blood flow during high-PEEP MV with pneumothorax was not different compared with low-PEEP MV (Fig. 4C). These data support the notion that high-PEEP MV may impede total diaphragmatic blood flow via mechanical compression of the diaphragm vasculature coupled with diaphragm inactivity.

**Medical costal diaphragm blood flow.** Given the heterogeneous distribution of blood flow in the diaphragm (3, 7, 28, 38), measurements of total diaphragm hemodynamics alone may not be sufficient to elucidate the effects of MV on diaphragm function. The medical costal diaphragm sustains the greatest proportion of diaphragm inspiratory work (28, 38) and may be more vulnerable to inactivity-mediated tissue and vascular injury (14). In the present investigation, both low- and high-PEEP MV significantly increased medical costal diaphragm vascular resistance, with concomitant reductions in blood flow (Fig. 3, A and B). In addition, medical costal diaphragm vascular resistance was significantly higher during high-PEEP MV versus low-PEEP MV (Fig. 4B). Therefore, acute MV (≤10

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**Table 2. Tissue vascular resistances and blood flows from low-PEEP and high-PEEP MV animals**

| Tissue          | SB (n = 10) | LP MV (n = 10) | LP MV + LAP (n = 7) |
|-----------------|-------------|---------------|-------------------|
| **Low PEEP**    |             |               |                   |
| Costal diaphragm|             |               |                   |
| Ventral         | 1.9 ± 0.4   | 2.8 ± 0.4     | 1.8 ± 0.1*        |
| Dorsal          | 1.6 ± 0.1   | 3.7 ± 0.7*    | 2.2 ± 0.3         |
| Crural diaphragm| 2.8 ± 0.5   | 4.3 ± 0.7     | 3.3 ± 0.5         |
| Soleus          | 6.0 ± 0.5   | 3.5 ± 0.7*    | 3.2 ± 0.4*        |
| Internal intercostal | 5.7 ± 1.6 | 9.0 ± 2.0 | 2.6 ± 0.3*        |
| External intercostal | 4.0 ± 0.8 | 8.5 ± 2.5 | 3.1 ± 0.8         |
| Kidney          | 0.15 ± 0.02 | 0.28 ± 0.04*  | 0.15 ± 0.02†      |

| **High PEEP**   |             |               |                   |
| Costal diaphragm|             |               |                   |
| Ventral         | 67 ± 12     | 48 ± 5        | 65 ± 6‡           |
| Dorsal          | 62 ± 6      | 44 ± 6**      | 62 ± 12           |
| Crural diaphragm| 51 ± 11     | 49 ± 6        | 51 ± 14           |
| Soleus          | 17 ± 2      | 49 ± 12*      | 41 ± 6†           |
| Internal intercostal | 28 ± 7 | 27 ± 4 | 49 ± 18*          |
| External intercostal | 31 ± 6 | 25 ± 6 | 47 ± 17†          |
| Kidney          | 738 ± 72    | 505 ± 38*     | 752 ± 80†         |

| Tissue          | SB (n = 9) | HP MV (n = 6) | HP MV + PTX (n = 6) |
|-----------------|-----------|---------------|---------------------|
| **Vascular resistance, mmHg/mL/min/100 g** |
| Costal diaphragm|           |               |                     |
| Ventral         | 2.8 ± 0.6 | 6.5 ± 1.3*    | 4.8 ± 1.8§          |
| Dorsal          | 1.7 ± 0.1 | 4.3 ± 0.5*    | 2.2 ± 0.3‡          |
| Crural diaphragm| 2.9 ± 0.5 | 7.8 ± 1.3*    | 4.6 ± 0.5‡          |
| Soleus          | 3.8 ± 0.5 | 6.8 ± 1.0*    | 4.6 ± 1.3           |
| Internal intercostal | 4.5 ± 0.4 | 9.3 ± 3.0    | 7.2 ± 2.0           |
| External intercostal | 4.6 ± 0.7 | 11.2 ± 2.0   | 14 ± 5.2**          |
| Kidney          | 0.14 ± 0.01| 0.30 ± 0.04*  | 0.15 ± 0.02‡        |

| Tissue blood flow, mL/min/100 g |
|-------------------------------|
| Costal diaphragm               |
| Ventral                       | 49 ± 13 | 20 ± 3* | 36 ± 9 |
| Dorsal                        | 57 ± 6  | 27 ± 2* | 52 ± 8‡|
| Crural diaphragm              | 37 ± 5  | 19 ± 4* | 23 ± 2‡|
| Soleus                        | 31 ± 3  | 23 ± 6  | 31 ± 7 |
| Internal intercostal          | 22 ± 2  | 23 ± 6  | 15 ± 3 |
| External intercostal          | 25 ± 4  | 16 ± 4  | 23 ± 11 |
| Kidney                        | 860 ± 112 | 464 ± 54* | 818 ± 114‡ |

Data are means ± SE; n, number of rats. HP MV, high-positive end-expiratory pressure (PEEP) mechanical ventilation; HP MV + PTX, high-PEEP mechanical ventilation + pneumothorax; LP MV, low-PEEP mechanical ventilation; LP MV + LAP, low-PEEP mechanical ventilation + laparotomy; SB, spontaneous breathing. *Significant (P ≤ 0.05) vs. SB. †Significant (P ≤ 0.05) vs. LP MV. #Nonsignificant (P = 0.1) vs. LP MV. **Significant (P ≤ 0.1) vs. SB; ‡Significant (P ≤ 0.05) vs. HP MV. §Nonsignificant (P ≤ 0.1) vs. HP MV.

**Diaphragm blood flow and vascular resistance.** During acute MV (e.g., 30 min) diaphragmatic blood flow is reduced (7, 15, 33, 43), and when MV is extended from 30 min to 6 h there is an additional ~75% reduction in diaphragm perfusion (7). In the present study, total diaphragm blood flow during low- and high-PEEP MV was significantly reduced within 10 min of MV (Fig. 2, C and D). Analogous to the reduced diaphragm vascular conductance previously demonstrated with MV (7), low- and high-PEEP MV increased diaphragm vascular resistance, which resulted in the reduced diaphragm perfusion (Fig. 2, A and B). Interestingly, high-PEEP MV increased vascular resistance and reduced blood flow in the whole diaphragm to a greater extent than low-PEEP MV (Fig. 4, A and C). The larger reduction in total diaphragm blood flow with higher PEEP may be related to a decrease in transmural pressure in the diaphragm vasculature during MV. This effect may arise from greater intrathoracic pressures and/or compressive effects on the thoracic aorta and possibly the phrenic artery, raising diaphragm arteriolar intravascular pressure and instigating myogenic vasoconstriction.

Given the heterogeneous distribution of blood flow in the diaphragm (3, 7, 28, 38), measurements of total diaphragm hemodynamics alone may not be sufficient to elucidate the effects of MV on diaphragm function. The medical costal diaphragm sustains the greatest proportion of diaphragm inspiratory work (28, 38) and may be more vulnerable to inactivity-mediated tissue and vascular injury (14). In the present investigation, both low- and high-PEEP MV significantly increased medical costal diaphragm vascular resistance, with concomitant reductions in blood flow (Fig. 3, A and B). In addition, medical costal diaphragm vascular resistance was significantly higher during high-PEEP MV versus low-PEEP MV (Fig. 4B). Therefore, acute MV (≤10
min), with low or high PEEP, results in significant reductions in medial costal diaphragm blood flow. The elevated vascular resistance with high versus low PEEP indicates either a more pronounced vasoconstriction or possibly compression of arterioles within the medial costal portion of the diaphragm. Importantly, a prolonged enhanced constriction, or compression, could lead to vascular entrenchment (22, 23) and accelerate the development of vascular dysfunction. This may explain mechanistically the inability to augment diaphragm blood flow after prolonged MV (7) and the associated microvascular dysfunction (13), which may be more pronounced with elevated PEEP. The rapid reductions in diaphragm perfusion, most apparent within the medial costal portion, may result in areas of local tissue hypoxia, supported by previously demonstrated elevations in HIF-1α in the diaphragm with MV (4). In addition, reduced diaphragmatic blood flow precipitates diaphragmatic fatigue (40). Furthermore, prolonged MV (i.e., 6 h) with zero PEEP elicits time-dependent reductions in diaphragm vascular conductance and blood flow (7). In rabbits, irrespective of PEEP setting, prolonged MV (i.e., 48 h) compromises diaphragm force generation (35). These data suggest that there may be a time-dependent threshold for diaphragm perfusion reductions with MV. In other words, the lowering of diaphragm blood flow during MV occurs regardless of the PEEP setting, and higher levels of PEEP could accelerate the reductions in diaphragm perfusion. Thus, with acute MV the reduced medial costal diaphragm blood flow is more pronounced with high-PEEP MV.

Recently, it has been suggested that the severe time-dependent reductions in diaphragmatic blood flow with prolonged MV are due to repetitive caudal displacement of the diaphragm and supraphysiological intrathoracic pressure generation (42). Although positive-pressure MV does alter intrathoracic pressure, there is no direct evidence that these pressure changes actually impede diaphragmatic blood flow. Interestingly, Bruells and colleagues (5) demonstrated that prolonged MV (i.e., 12 h), independent of the manner in which tidal volume is delivered (i.e., negative- vs. positive-pressure ventilation), results in similar levels of diaphragm dysfunction. In the present study, ablation of intra-abdominal and intrathoracic pressures during MV both significantly lowered vascular resistance and augmented medial costal diaphragm blood flow versus high-PEEP MV (Fig. 4, B and D). However, the lower medial costal diaphragm vascular resistance and higher blood flow with laparotomy and pneumothorax were not different compared
with low-PEEP MV. Thus, our data suggest that diaphragm inactivity during MV, independent of PEEP setting, is the initial cause of the reduced medial costal diaphragm blood flow.

If the reductions in diaphragmatic blood flow and oxygen delivery during prolonged MV (7) were due to caudal diaphragm displacement and intrathoracic pressure generation, then upon removal of MV diaphragm blood flow should increase in direct proportion to the increased metabolic demand of spontaneous breathing during weaning. However, after prolonged MV (i.e., 6 h), there is an inability of the diaphragm to augment blood flow in response to contractions (7). Therefore, the reductions in diaphragmatic blood flow and vascular impairments with prolonged MV (13) cannot be solely attributed to the pressures in the thoracic cavity. The data presented here suggest that the MV-induced reductions in diaphragmatic blood flow are due to increased vascular resistance in the quiescent diaphragm. In addition, the decreased renal perfusion with low- and high-PEEP MV is due to increased renal vascular resistance.

Importantly, MV with PEEP can result in PEEP-induced reflex activation of the sympathetic nervous system and increased renal sympathetic activation through the offloading of cardiopulmonary low-pressure baroreceptors (37). Given the increase in HR and MAP with low- and high-PEEP MV (Table 1), pulmonary stretch receptors with vagal afferents (i.e., pulmonary A fibers) may also be involved in the sympathetic activation during MV with PEEP. In dogs, low levels of static lung inflation induce excitatory reflex tachycardia and vasoconstriction (1, 12, 16). This reflex activation is likely mediated by pulmonary A fibers, which demonstrate an airway pressure threshold for activation at ~5 cmH2O (17). This PEEP-induced sympathoexcitation can result in increased plasma norepinephrine, increased renin activity, and subsequent angiotensin II (ANG II) formation (2, 36, 37), which may explain the increased renal vascular resistance demonstrated here. The increase in diaphragm vascular resistance may be related to increased levels of catecholamines and/or ANG II, which can desensitize both α1- and angiotensin II (AT1)-mediated smooth muscle contraction (6, 19, 39). If the diaphragm vasculature is exposed to increased levels of circulating catecholamines in vivo during prolonged MV, it is possible to have increased vascular resistance and α-AR desensitization simultaneously. Interestingly, prolonged MV can result in α-AR desensitization and reduced α1-AR mediated vascular control in the diaphragm (13). During weaning, a reduced α-AR sensitivity will decrease the vasoconstrictive impact of increased circulating catecholamines in vivo. However, both impaired endothelial function and reduced nitric oxide (NO) bioavailability would compromise the ability of the diaphragm vasculature to augment blood flow with increased metabolic demands (7, 13). Therefore, increased levels of circulating catecholamines and/or ANG II may play a role in the increased diaphragm vascular resistance during MV and the inability to augment blood flow during weaning.

**Experimental considerations.** Diaphragm quiescence was not verified directly in our investigation. However, during prolonged controlled MV in the anesthetized rat (end-tidal CO2 presumably ≤ 30 mmHg), the diaphragm is inactive as verified through electromyography (30, 34). Since end-tidal CO2 was maintained at or below 30 mmHg throughout the MV protocol here, it was unlikely that there was any compelling chemoreceptor-induced drive to breathe. Diaphragm inactivation was verified visually in each experiment.

**Ramiﬁcations.** PEEP is used during MV in critically ill patients to improve oxygen exchange, with higher levels of PEEP employed in severely hypoxemic patients, such as critically ill ARDS and COVID-19 patients (29). It is postulated that MV-induced diaphragmatic dysfunction is initiated by diaphragm inactivity (31). Furthermore, reduced diaphragmatic blood flow promotes diaphragm muscle fatigue (40), and diaphragm ischemia-reperfusion results in a downward shift in the force-frequency relationship (41). The augmented vascular resistance and rapid reductions (≤ 10 min) in diaphragm perfusion with low- and high-PEEP MV demonstrated here may facilitate diaphragm ischemia and oxidative stress at some point after the onset of MV. Thus, the reduction in total and medial costal diaphragm blood flow during acute MV, with low and high PEEP, may predispose patients to diaphragm ischemia-reperfusion injury and prolonged weaning and complicate long-term diaphragm health.

**Conclusions.** Acute MV (i.e., ≤10 min), independent of PEEP setting, increases diaphragm vascular resistance, and reduces bulk diaphragmatic blood flow and regional diaphragmatic blood flow distribution. Future studies are needed to investigate the possible long-term consequences of varying PEEP levels on diaphragm perfusion and vascular function. We predict that prolonged MV with higher levels of PEEP may accelerate diaphragm blood flow reductions and exacerbate the MV-induced diaphragm vascular dysfunction.

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**DISCLOSURES**

No conflicts of interest financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

A.G.H., C.S.B., T.I.M., D.C.P., and B.J.B. conceived and designed research; A.G.H. and D.R.B. performed experiments; A.G.H. analyzed data; A.G.H., D.R.B., T.I.M., D.C.P., and B.J.B. interpreted results of experiments; A.G.H. prepared figures; A.G.H. drafted manuscript; A.G.H., D.R.B., K.M.S., O.N.K., T.D.C., R.E.W., C.S.B., T.I.M., D.C.P., and B.J.B. edited and revised manuscript; A.G.H., D.R.B., K.M.S., O.N.K., T.D.C., R.E.W., C.S.B., T.I.M., D.C.P., and B.J.B. approved final version of manuscript.

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