The effects of prolonged mechanical ventilation on the alpha-adrenergic responses of costal diaphragm arterioles

by

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Abstract

Introduction: Mechanical ventilation (MV) is a life-saving intervention employed in Intensive Care Units worldwide. Previous work has shown a substantial time-dependent reduction in diaphragmatic blood flow and O_2 delivery with MV, likely contributing to diaphragm fatigue and weaning difficulties. However, the precise mechanism(s) responsible for these alterations in blood flow are unknown. Given the reduction in diaphragmatic blood flow with prolonged MV, we tested the hypothesis that prolonged MV (e.g. 6 hours) enhances diaphragm arteriole contractile responses to alpha-adrenergic agonists with no change in sensitivity.

Methods: Female Sprague Dawley rats (5-8 mo) were randomly divided into spontaneous breathing (SB, n = 9) and prolonged MV (6 h MV, n = 6) groups. Following SB and 6 h MV, diaphragm arterioles (~200 μm diameter, ~2 mm in length) were isolated, cannulated, and pressurized to develop spontaneous tone. Thereafter, contractile responses to cumulative doses of non-selective α-adrenergic receptor agonist, norepinephrine (NE), and α_1-adrenergic receptor specific agonist phenylephrine (PE) (10^{-9} to 10^{-4}M) were determined.

Results and Conclusion: Prolonged MV did not alter maximal vasoconstrictor responses to NE but reduced PE-induced vasoconstriction (SB, 37.3 ± 6.7 vs. MV, 19.0 ± 1.9%; $p \leq 0.05$). Sensitivity (EC_{50}; concentration of a drug needed to elicit half-maximal response) was significantly reduced following prolonged MV, in response to NE ($p \leq 0.05$) and PE ($p \leq 0.05$). Following 6 h of MV, the reduction in α–adrenergic responsiveness occurs through both endothelial-dependent and –independent mechanisms, and likely contributes to the severe reductions in diaphragmatic blood flow and O_2 delivery during MV. Future studies are warranted, investigating responses to other vasoactive mediators such as Angiotensin-II and Endothelin-1, as well as potential alterations in vascular mechanical and material properties, with MV.

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Dedication

To Darla Jean Horn and Nick Ewing, whose selfless and vivacious characters will always be my source of inspiration. I also want to dedicate this thesis to my parents, Richard Horn and Leslie Lower, my stepfather, Robert Lower, and my brothers, Woody and Samuel Horn; none of this would have been possible without your support.

Chapter 1 - Literature Review

The Diaphragm

The diaphragm is a unique, dome-shaped skeletal muscle that is continuously active throughout life and accounts for the largest amount of work in mammalian ventilation (66). Serving as a barrier between the thoracic and abdominal cavities, the diaphragm arises from three embryological sources: the septum transversum, pleuroperitoneal folds, and somites (51). The septum transversum is a thin mesodermal sheet of tissue, separating the heart and the liver and likely serves as scaffolding for diaphragm morphogenesis during development (51). The pleuroperitoneal folds are two evanescent, pyramidal-shaped structures that extend from the body wall between the pleural and peritoneal cavities, located on either side of the esophagus (51) and fuse with the septum during development (3, 32). The somites are the source of diaphragm muscle cells (3, 19, 5). Specifically, diaphragm muscle progenitor cells likely originate from C3-C5 cervical somites, then proliferate and differentiate to form the costal and crural diaphragm (3, 19, 5). While only accounting for ~0.5% of body weight, the diaphragm displays a higher oxidative capacity compared to most skeletal muscles and is comprised of 44% type I, 6% type IIA, 18% type IIX, and 32% type IIB muscle fibers (17, 68). The diaphragm is divided into two distinct muscle regions that extend outward from the central tendon: the costal can be subdivided into 3 sections (ventral, medial, and dorsal) that attach to the lower ribs at the costal margin, and a crural section (most dorsal) attached at the lower ribs and lumbar vertebra (Figure 1.1).

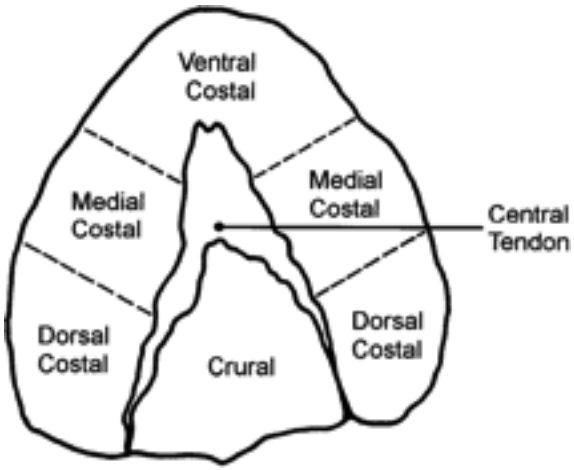


Figure 1.1 Schematic of the rat diaphragm showing the crural, dorsal, medial, and ventral costal diaphragm sections. (From reference 49. Poole et al., Diaphragm structure and function in health and disease. Med Sci Sports Exerc 29: 738-754, 1997)

The phrenic artery bilaterally enters the diaphragm at the crural section, and then diverges into its arterial tree to provide blood supply to the entire diaphragm, with the medial costal diaphragm receiving the highest mass specific blood flow (82). There are regional differences (i.e. ventral costal versus medial costal portion) in diaphragm muscle fiber thickness (48, 47, 99), blood flow distribution (82), and differential vasomotor regulation of the arterial network (1). Further, the diaphragm possesses a large functional vasodilator reserve at both rest (quiet breathing) and maximal exercise (65), and an impressive capacity for blood flow; increasing ~6x from rest to maximal exercise (4). To coincide with the enormous capacity for blood flow, the diaphragm demonstrates a specialized microcirculation (e.g., countercurrent capillary flow; (37)) with a capillary density, length, volume, and surface area per fiber volume that is 2- to 3-fold greater compared to other hindlimb muscles (29, 64), structurally and functionally optimized to facilitate oxygen transfer (66). Although continually active throughout life, the diaphragm can become fatigued during exercise (34) and in chronic diseases such as emphysema (63), diaphragm exhaustion faciltates respiratory muscle failure. The diaphragm also exhibits a high

level of plasticity (66) and undergoes distinct adaptations in order to meet physiological and pathophysiological metabolic demands.

Oxygen and diaphragm function

In 1604, Polish alchemist Michael Sendivogius (1566-1636) published that air contains what he deemed the "secret food of life (109)." This "food" was oxygen; by heating saltpetre (potassium nitrate), Sendivogius had beautifully synthesized oxygen almost 170 years prior to the monumental contributions to the field of chemistry by Carl Wilhelm Scheele (1742-1786), Joseph Priestley (1733-1804), and Antoine-Laurent de Lavoisier (1743-1794). Oxygen is an element vital for life and is regarded as the most important discovery in chemistry (108).During inspiration, oxygen is transported from the atmosphere to the mitochondria to facilitate oxidative adenosine triphosphate (ATP) synthesis. Occurring within the inner mitochondrial membrane, oxidative phosphorylation is essential for energy metabolism. Oxidative phosphorylation is a process coupled to the oxidation of hydrogen ions (H^+) (via the respiratory chain), nicotinamide adenine dinucleotide ($NADH+H^+$), and flavin adenine dinucleotide ($FADH_2$). The mitochondrial respiratory chain is a series of reduction and oxidation reactions within complexes I, II, III, and IV, which are linked by ubiquinone (Q) and cytochrome c (cyto-c). Ubiquinone is reduced to ubiquinol (QH_2), and shuttles electrons from both complex I and II to complex III, while cyto-c shuttles electrons from complex III to IV. It is at complex IV where the electrons shuttled through the respiratory chain are donated to molecular O_2, which is then reduced to H_2O. This simplified process of oxidative phosphorylation can be represented by the Phosphate/Oxygen (P/O_2) ratio:

$$\underline{6}ADP + \underline{6}P_i + \underline{2}(NADH+H^+) + \underline{1}O_2 \longrightarrow \underline{6}ATP + \underline{2}NAD^+ + \underline{2}H_2O$$

According to the P/O_2 ratio, for every 1 molecule of oxygen, 6 ATP molecules are synthesized at the site of respiration in the mitochondria. However, it should be noted that due to membrane leak, the P/O_2 ratio, *in vivo*, is lower; i.e., ~3.5 for succinate, and ~5.5 for NADH oxidation (6).

Any disturbance in the oxygen cascade, from atmospheric air to the mitochondria, will result in a reduction of overall O_2 transport, potentially, skeletal muscle fatigue (98). To fully grasp the mechanisms responsible for skeletal muscle fatigue, specifically within the diaphragm, it is pertinent to assess O_2 flux close to the site of O_2 utilization, i.e., the microcirculation. Microvascular oxygenation ($P_{mv}O_2$), governed by the balance of O_2 delivery ($\dot{Q}o_2$) to O_2 consumption ($\dot{V}o_2$), is the driving pressure for blood to myocyte O_2 delivery to sustain cellular respiration and skeletal muscle metabolic demands. Poole and colleagues (67), were the first to demonstrate phosphorescence quenching as a practical tool to measure diaphragm $P_{mv}O_2$ *in vivo*. Within the diaphragm (and other skeletal muscle), in order to sustain the metabolic and contractile function, there must be a tight relationship between the rate of $\dot{Q}o_2$ and $\dot{V}o_2$ (63). Diaphragm and other skeletal muscle $\dot{V}o_2$ is the product of diaphragm $\dot{Q}o_2$ and fractional O_2 extraction. In regard to diaphragm $\dot{V}o_2$, muscle O_2 extraction is not determined by blood flow ($\dot{Q}$) or O_2 diffusing capacity (Do_2), *in seperatum*, but instead through the balance between $\dot{Q}$ and Do_2 (74). Given the large functional reserve of blood flow capacity of the diaphragm previously mentioned, one would hypothesize that this specialized respiratory muscle would be particularly protected from deficits in blood flow. However, as discussed below, with 30 m and 6 h MV, there are significant time-dependent reductions in diaphragm $P_{mv}O_2$, $\dot{Q}o_2$, and $\dot{V}o_2$, at rest and during contractions (Figure 1.2.; (16)). Importantly, other conditions, such as increased α-adrenoceptor activation, have been demonstrated to result in an $\dot{Q}o_2$ to $\dot{V}o_2$ mismatch in the diaphragm, through an O_2 delivery limitation (100).

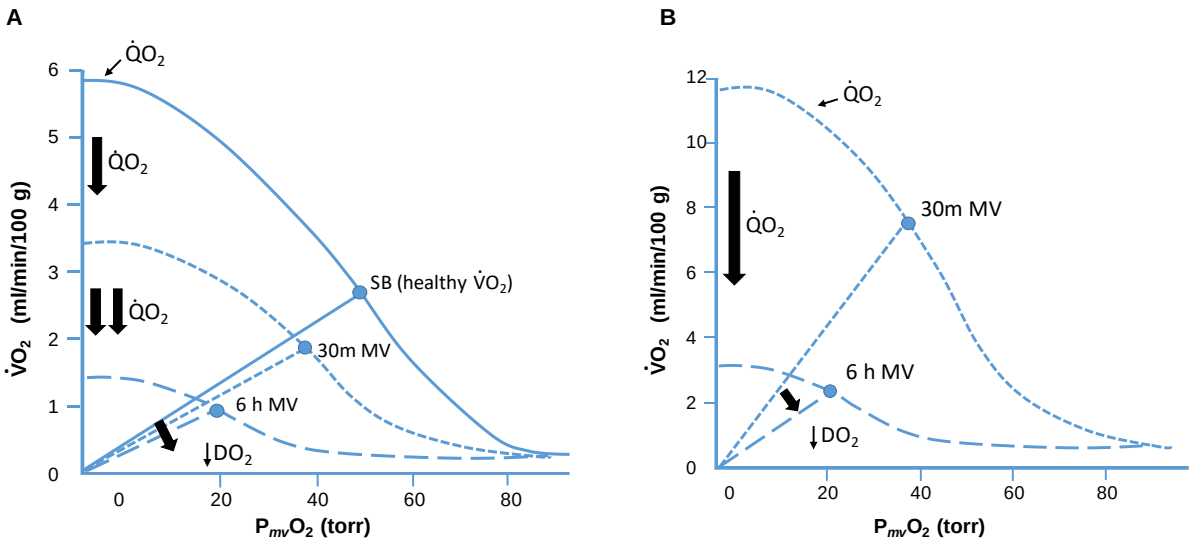


Figure 1.2. Schematic representations of muscle O_2 uptake ($\dot{V}O_2$) plotted as a function of microvascular PO_2 ($P_{mv}O_2$) at rest (A) and 1Hz contractions (B). Merging of the Fick principle, perfusive (*curved line*), and Fick's Law, diffusive (*straight line from the origin*) O_2 delivery allows for a mechanistic analysis of the $\dot{V}O_2$ achieved in health, 30 m MV, and 6 h MV.

The structure and function of arterioles

Arterioles consist of three distinct layers: tunica intima, media, and adventitia. The tunica intima, the most inner layer, is comprised of endothelial cells arranged longitudinally and an abluminal basement membrane. In response to increases in blood flow (and shear stress), the endothelium releases vasoactive substances (e.g., nitric oxide) that regulate vessel diameter and can influence the mechanical function of the vessel wall (49). In arterioles, the basement membrane beneath endothelial cells (~0.1 μm thick) serves to anchor and support the endothelium and is primarily made up of type IV collagen, laminin, and heparan sulfate proteoglycans (72). The middle layer, tunica media, is the mechanical controller of vessel diameter, comprised of vascular smooth muscle cells (VSMC) and internal elastic lamina. The internal elastic lamina (~0.3 μm thick), is composed of degradation-resistant elastin molecules (72). Present mainly in the feed arterioles of skeletal muscle (72, 106), the internal elastic lamina

provides recoil properties to the vessel wall, which permits arterioles to accommodate pulsatile blood flow (106). The most abundant portion of the tunica media is the smooth muscle layer, which modulates vessel diameter via contraction and relaxation in response to various stimuli. VSMC (~100 μm in length) are arranged in circumferential fashion, perpendicular to the longitudinal axis of the vessel (23, 53) and react to mechanical forces (i.e. shear stress) through mechanotransduction (49). The most superficial layer, tunica adventitia, is a collagen fiber rich matrix orientated along the longitudinal axis of arterioles (71, 78) and aids in anchoring nerve endings that act on VSMC. Elastic fibers are also present in the adventitia, as well as fibroblasts which are embedded in the collagen fiber rich matrix (49). The elastic fibers allow resistance arterioles to elongate and recoil longitudinally in tissues such as skeletal muscle.

Arterioles can account for up to 80% of vascular resistance in skeletal muscle and other organs and are vital to blood pressure regulation and the distribution of cardiac output; dilating or constricting to regulate the blood flow through capillaries in various vascular beds (49). The diameter of arterioles is regulated by local factors (i.e. metabolites), circulating factors (i.e. hormones), and the sympathetic nervous system. The regulation of peripheral blood flow is under dual control: centrally, by the nervous system, and locally in the tissue, by the physiochemical conditions present in the immediate region of blood vessels. Vascular smooth muscle contraction (vasoconstriction) is mediated by Ca^{++} influx through voltage-gated channels (L-type) and Ca^{++} release from the sarcoplasmic reticulum, via the myogenic response (i.e. increased transmural pressure) and endogenously released vasoactive substances from the endothelium (e.g., norepinephrine, angiotensin-II, endothelin-1) (107). The endothelium plays a fundamental role in setting vascular tone, by synthesizing and releasing vasodilatory factors (i.e. NO, PGI_2, EDHF), and vasoconstrictor factors (i.e. TXA_2, ET-1). In addition, the endothelium is

largely involved in the hyperemic response of skeletal muscle by facilitating vasodilation to augment blood flow to the muscle during increased metabolic activity. Alterations in vascular homeostasis, acutely and chronically, can improve (i.e. exercise) or impair vessel function (i.e. hypertension) and, importantly, changes in mechanical stimuli (i.e. reduction in blood flow or shear-stress) that occur in many pathologies can promote physiological adaptations or disease of the vessel wall (15).

Alpha-adrenergic receptor signaling

Alpha-adrenergic receptors (α-AR) are G-protein coupled receptors (GPCRs) that participate in various essential physiological processes, such as sympathetic neurotransmission, vascular tone, cardiac contractility, and smooth muscle contraction (39). In 1948, after investigating the control of systemic blood pressure, regional blood flow, and myocardial contractility with various agonists, before and after dibenamine (irreversible α_1-AR blocker) treatment, Raymond Alquist (1914-1983) proposed dividing adrenergic receptors into two classes: α and β. Following the subclassification of adrenergic receptors (2), α-ARs were further divided into α_1- and α_2-ARs based on the relative potency of selective α-AR agonists and antagonists (7, 40). Initially, α_2-ARs were identified as a presynaptic inhibitory receptor (40, 87) and a postjunctional receptor on smooth muscle cells (7), while α_1-ARs were proposed as a postjunctional receptor that mediated the effector organ (excitatory) response (40). Thus, Berthelsen and Pettinger (7), proposed a functional classification of α-adrenoceptor subtypes, rather than anatomical classification, where α_1-ARs mediated excitatory responses and α_2-ARs mediated inhibitory responses. However, in 1979, Drew and Whiting (20) demonstrated that norepinephrine (NE) mediated vasoconstriction through both α_1- and α_2 -ARs, which resulted in the universally accepted pharmacological receptor subclassification, based on relative affinities

of selective antagonists and agonists. Following the development of the radioligand binding assay, α_1-ARs (26, 56) and α_2-ARs (13) were further divided into α_1- (α_{1A}, α_{1B}, α_{1D}) and α_2– (α_{2A}, α_{2B}, α_{2C}) subtypes.

In the peripheral circulation, catecholamines (i.e. norepinephrine, epinephrine) elicit physiological effects on a target tissue by binding to alpha (α_1 and α_2) and beta (β_2) adrenergic receptors on VSMCs. In addition to VSMCs, α_2-ARs are expressed on endothelial cells and, in conjunction with other receptors (i.e. P_2Y, $\mathcal{M}_3$, BK_R), mediate endothelial-dependent vasodilation (10, 84). α_2-AR agonists (i.e. NE) bind to α_2-ARs and activate the coupled G-protein (G_i), which then activates phospholipase C (PLC), followed by the increased release of endothelial-derived relaxing factor (EDRF; Figure 1.3). Endothelial α_2-ARs also mediate vasorelaxation through endothelial-nitric oxide synthase (eNOS) activation, thus nitric oxide (NO) synthesis, and prostacyclin (PGI_2) production via increased inositol triphosphate (IP3) turnover and intracellular calcium release (Ca^{2+}) (Figure 1.3). NO then activates soluble guanylate cyclase (sGC), increasing cyclic guanosine monophosphate (cGMP) and PGI_2 binds to prostacyclin receptors, elevating cyclic adenosine monophosphate (cAMP); the increases in cGMP and cAMP in VSMCs cause vasodilation (Figure 1.3).

In skeletal muscle, NE is released from sympathetic nerve terminals and non-selectively binds to α_1- and α_2-ARs to elicit VSMC contraction and, consequently, reduces blood flow (77, 46). The phosphorylation of myosin light chain 20 (MLC_{20}) is key determinant for VSMC contraction and is regulated by the activity ratio between myosin light chain kinase (MLCK) and myosin phosphatase (MP). The activity of MLCK is modulated by the cytosolic Ca^{2+} concentrations, while MP activity is controlled via upstream signaling molecules (e.g. RhoA) (28). When an agonist binds to α_1-ARs, the coupled G-protein (G_q) activates phospholipase C

(PLC), which stimulates phosphatidylinositol 4,5-bisphosphate (PIP2) to release diacylglycerol (DAG) and inositol triphosphate (IP3). DAG then stimulates cytosolic protein kinase C (cPKC) and IP3 stimulates intracellular Ca^{2+} release from the sarcoplasmic reticulum (SR). The increases in intracellular Ca^{2+} from the SR and extracellular influx through L-type calcium channels, along with calmodulin (CaM), stimulate MLCK activity and initiate VSMC contraction and vasoconstriction (Figure 1.4). It is important to note that various agonists (i.e. ET-1, VP, AT-II) and their respective receptors, also participate in VSMC contraction and vasoconstriction (Figure 1.4). Conversely, when an agonist (i.e. NE) binds to α_2 -ARs, the G-protein (G_i) is activated and reduces adenylate cyclase (AC) activity. The reduced AC activity lowers cyclic adenosine monophosphate (cAMP) concentrations and reduces the inhibitory effect on MLCK, thus, facilitating MLC_{20} phosphorylation and VSMC contraction (Figure 1.4). Although not discussed herein, it has been shown that α_1-AR activation induces reactive oxygen species (ROS) formation that may participate in the regulation of α_1-AR mediated vasoconstriction (91). Tissue transglutaminase-2 (TG2) has also been proposed to be involved in α_1-AR activation, by acting as a G-protein in the signal transduction (58).

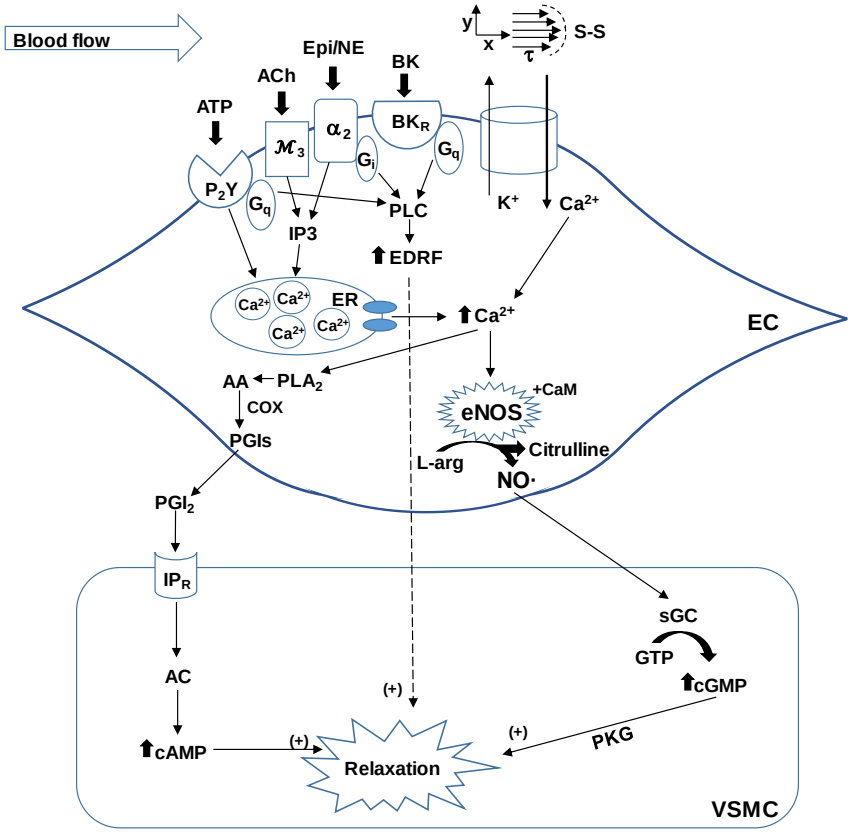


Figure 1.3. Schematic representation of signaling pathways involved in endothelial cells (EC) and their interaction with vascular smooth muscle cells (VSMC) in vasodilation. It is important to note that there are other signaling pathways arising in endothelial cells that mediate vasoconstriction (i.e. endothelin-1, angiotensin-II, thromboxane A_2, endoperoxides) which are not illustrated here. AA; *arachidonic acid*, AC; *adenylate cyclase*, ACh; *acetylcholine*, ATP; *adenosine triphosphate*, α_2; *alpha-2 adrenoceptor*, BK; *bradykinin*, BK_R; *bradykinin receptor*, Ca^{2+}; *calcium ion*, CaM; *calmodulin*, cAMP; *cyclic adenosine monophosphate*, cGMP; *cyclic guanosine monophosphate*, COX; *cyclooxygenase*, EDRF; *endothelial-derived relaxing factor*, eNOS; *endothelial nitric oxide synthase*, Epi; *epinephrine*, ER; *endoplasmic reticulum*, G_i; *adrenoceptor coupled G-protein*, G_q; *bradykinin and purigenic receptor coupled G-protein*, GTP; *guanosine triphosphate*, IP3; *inositol triphosphate*, IP_R; *prostacyclin receptor*, K^+; *potassium ion*, L-arg; *L-arginine*, $\mathcal{M}_3$; *muscarinic receptor*, NO·; *nitric oxide*, NE; *norepinephrine*, P_2Y; *purigenic receptor*, PGI_2; *prostacyclin*, PKG; *protein kinase G*, PLA_2; *phospholipase A*$_2$, PLC; *phospholipase C*, sGC; *soluble guanylate cyclase*, S-S; *shear stress*, τ; *shear rate acting on endothelial cell surface*, (+); *stimulus for vasorelaxation*. This schematic was created based on previous research discussed in the literature review herein (94, 95, 75).

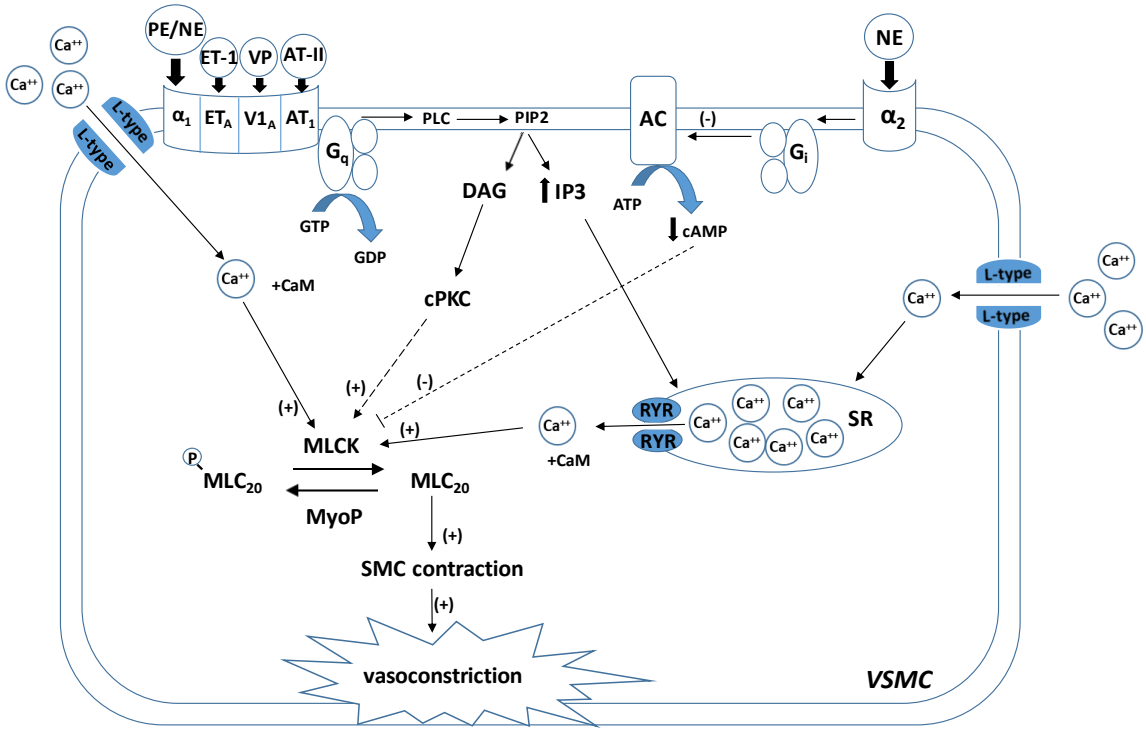


Figure 1.4. A schematic representation of the alpha-adrenergic and other pathways, involved in vascular smooth muscle cell contraction. AC*; adenylate cyclase,* ATP*; adenosine triphosphate,* AT_1*; angiotensin receptor,* AT-II*; angiotensin-II,* α_1 *; alpha-1 adrenoceptor,* α_2*; alpha-2 adrenoceptor,* Ca^{++}*; calcium ion,* CaM*; calmodulin,* cAMP*; cyclic adenosine monophosphate,* cPKC*; cystolic protein kinase C,* ET-1*; endothelin,* DAG*; diacylglyceride,* ET_A*; endothelin receptor,* G_i *and* G_q*; g-protein complexes coupled to respective alpha-adrenoceptor,* GDP*; guanosine diphosphate,* GTP*; guanosine triphosphate,* IP3*; inositol triphosphate,* L-type*; voltage-gated calcium ion channel,* MLC_{20}*; active myosin light chain 20,* MLC_{20} +P*; inactive myosin light chain 20,* MLCK*; myosin light chain kinase,* MP*; myosin phosphatase,* NE*; norepinephrine,* PE*; phenylephrine,* PLC*; phospholipase C,* PIP2*; Phosphatidylinositol 4,5-bisphosphate, RYR; ryanodine receptor,* SMC*; smooth muscle cell,* VSMC*; vascular smooth muscle cell,* $V1_A$*; vasopressin receptor,* VP*; vasopressin,* (+)*; positive effect,* (-)*; inhibitory effect.* This pathway schematic was constructed based on previous research discussed in the literature review herein (77, 105, 61, 91).

The Impact of Mechanical Ventilation and Ventilator-Induced Diaphragmatic Dysfunction on Diaphragm Function

An essential tool in surgery and intensive care units, mechanical ventilation (MV) is a life-saving intervention to sustain pulmonary gas exchange in patients who are incapable of maintaining sufficient alveolar ventilation (e.g. patients with respiratory failure, spinal cord injury, and heart valve replacement). More than half of patients admitted to the ICU are ventilated for the first 24 hours of admittance (110), and the frequency of MV is increasing >2% annually (59). In addition, the use of MV incommensurably drives resource use in the ICU, partly due to weaning complications, and results in ~70% of the aggregate health care cost of patients needing MV (~$16 billion annually in the US (104). Once MV is no longer needed, patients should meet certain subjective (i.e. adequate cough, absence of excessive trachea-bronchial secretion), objective (i.e. stable cardiovascular status, adequate hemoglobin levels), and adequate oxygenation (i.e. PaO_2 ≥60 mmHg) criteria to determine if MV can be successfully terminated (101). Weaning is the process of decreasing the amount of ventilatory support the patient receives, through spontaneous breathing trials (SBT), and the patient assumes a greater proportion of the ventilatory work; which accounts for 40% of the time spent on the ventilator (22). However, approximately 30% of patients encounter weaning difficulties, leading to more time spent on the ventilator (21, 11). When a patient fails the weaning procedure, they must undergo reintubation, which is associated with increased mortality rates (24). In humans, prolonged MV (≥18h) induces the rapid development of diaphragm fatigue and dysfunction (86), which plays a key role in the inability to wean patients from the ventilator.

Within pre-clinical animal models, prolonged controlled mechanical ventilation (CMV), where the diaphragm is mechanically inactive (70, 80), recapitulates the human ICU MV

conditions. Since the diaphragm is responsible for ~75% of the work of breathing during normal ventilation at rest (66), prolonged periods of disuse such as MV, are detrimental to diaphragm muscle function. MV-induced diaphragm injury was first identified in animal studies by Le Bourdelles et al. (41) and later confirmed in humans by Levine et al. (44). The etiology of failure to wean from MV, is due in part to diaphragm dysfunction, given the moniker Ventilator-induced Diaphragmatic Dysfunction (VIDD). VIDD is characterized by diaphragm atrophy, oxidative stress, muscle fiber remodeling, and mitochondrial dysfunction (96). Shanely and colleagues (83), demonstrated a reduction in protein synthesis in as little as 6 hours of MV, and current scientific opinion is that the rapid onset of diaphragmatic atrophy is primarily due to an increase in proteolysis (83, 69, 50). Several lines of work identify increased proteolysis and diminished protein synthesis as the key mechanisms involved in weaning failure and VIDD (33). Specifically, increased levels of reactive oxygen species (ROS) and nuclear factor kappa beta ($NF\kappa\beta$) pathway activity results in augmented levels of caspase and calpain proteins and ubiquitin-proteasome activity, which are attributed to the increased proteolysis (33). In addition to upregulated proteolysis with MV, decreased protein kinase B (Akt) phosphorylation and elevated lysosome-autophagy lead to reductions in protein synthesis (33).

During acute MV (i.e. 30 min), there is a significant reduction in blood flow to the diaphragm (73, 97, 31, 93). Prolonged MV (6 h) results in severe time-dependent reductions in diaphragmatic blood flow and microvascular oxygenation ($P_{mv}O_2$) at rest, and an inability to augment blood flow to the diaphragm in response to contractions (16). Further, reduced arteriolar vasodilation appears to play a key role in the attenuated diaphragmatic blood flow during and after MV (30). Reductions in oxygen delivery with prolonged MV may lead to reactive oxygen species (ROS) formation in combination with the activation of proteolytic cascades thus,

exacerbating oxidative stress (102). Bruells et al. (12), showed that 24 h of MV resulted in a downregulation of vascular endothelial growth factor (VEGF) and upregulation of hypoxia-inducible factor-1α (HIF-1α), which may be due to elevated inflammatory cytokine signaling via NFκβ pathway activation (12). Importantly, the effects of prolonged MV do not appear to be due to anesthesia, as there are no changes seen in other 'inactive' muscles, like the soleus, which does not demonstrate alterations in muscle mass (70) or blood flow (16) during MV. Interestingly, it appears that the diaphragm relies heavily on or at least upregulates glycolytic metabolism during weaning, as there is a five-fold increase in diaphragm venous blood lactate, a ~30% decrease in diaphragm glycogen stores, and a 50% reduction in [ATP] and [CP] (45).

There is a paucity of data regarding viable therapeutic interventions to improve weaning outcomes. Supinski et al. (88), reversed diaphragm fatigue through phrenic artery hyper-perfusion, which demonstrates the importance of diaphragmatic blood flow and the hyperemic response in mitigating diaphragm weakness. During MV, Gayan-Ramirez and colleagues (25), improved diaphragm force development (compared to MV alone) by utilizing intermittent spontaneous breathing, which may have inadvertently demonstrated the benefits of maintaining blood flow to the diaphragm during MV. Some data suggests that antioxidant therapy can attenuate diaphragm injury with MV (8, 50), albeit chronic antioxidant therapies do not improve patient mortality and may worsen patient prognosis (9). Further, there are no data upon how antioxidant treatment impacts diaphragm blood flow during MV. Theophylline, a widely prescribed bronchodilator for COPD patients, may have some potential to improve successful weaning but evidence is inconclusive due to confounding factors (i.e. reason for ICU admission, severity of illness) (85) and is often withheld from administration due to adverse side effects (92).

Chapter 2 - Introduction

Mechanical ventilation (MV) is a life-saving intervention to sustain pulmonary gas exchange in patients who are incapable of maintaining sufficient alveolar ventilation (e.g. patients with respiratory failure, spinal cord injury, and heart valve replacement); with the frequency of MV use increasing by over 2% annually (59). Prolonged MV in humans (≥18h) and ≥6 h in the pre-clinical animal model (59, 35, 70, 79, 86) induces diaphragm atrophy, contractile dysfunction, oxidative stress, and mitochondrial dysfunction. Collectively these perturbations, occurring primarily with prolonged MV, are known as ventilator-induced diaphragmatic dysfunction (VIDD; (96)). The development of VIDD is believed to be a key culprit in the inability to wean patients from the ventilator and is a major clinical problem as ~30% of patients experience weaning difficulties (11, 22) resulting in additional time on the ventilator and increased patient mortality (22, 24).

The precise physiological mechanisms responsible for VIDD, whilst unclear, are likely to be multifaceted. Previous studies have indicated increased reactive oxygen species (ROS) production, mitochondrial damage, skeletal muscle atrophy, and muscle fiber remodeling as potential contributors to diaphragm weakness and VIDD (24, 35, 70, 79, 86). One aspect of VIDD that has not been explored is the potentially altered vasomotor control of the resistance vasculature within the diaphragm (i.e. arterioles). In this regard, it is established that during acute MV (e.g. 30 min) blood flow to the diaphragm is reduced (31, 73, 93). Recently, our group has expanded these observations, demonstrating an additional reduction in diaphragmatic blood flow when MV is extended from 30 min to 6 h (16). The mechanistic bases for this time-dependent reduction in diaphragmatic blood flow with MV are unknown. The inability to augment diaphragm blood flow with contractions after prolonged MV (16), suggest a reduced

dilatory capacity and/or enhanced contractile responses of diaphragm resistance vessels occurring with prolonged MV. Importantly, this time-dependent reduction in blood flow with inactivity is not observed in other highly-oxidative muscles (e.g., the soleus and red portion of the gastrocnemius muscle) (16), suggesting that the diaphragm vasculature may be particularly vulnerable to inactivity-induced rapid vascular dysfunction.

Vasomotor control demonstrates considerable temporal plasticity. For example, Hill and colleagues (27), demonstrated that acute functional remodeling of resistance arterioles can occur during prolonged (4 h) exposure to alpha-adrenergic agonists (NE). In mice, chronic reductions in blood flow results in excessive medial and adventitial proliferation and negative hypertrophic remodeling (38, 76). Further, prolonged MV blunts diaphragm arteriolar relaxation (30) and the impairment of vasodilatory pathways has been shown to induce inward remodeling of resistance vessels (62). However, there is a paucity of data regarding the ramifications of reductions in blood flow on vasomotor function in chronically active muscle such as the diaphragm. There is evidence, from long-term (i.e., days/weeks) disuse, for a down-regulation of vasodilatory pathways in skeletal muscle (18).

The overall objective of this study was to investigate the effects of prolonged MV (i.e. 6 h), using an established pre-clinical animal model, on diaphragm resistance arteriole function. In resistance vessels from the diaphragm of animals subjected to prolonged MV versus spontaneous breathing, we tested the hypotheses that there will be: 1) an increased alpha-adrenergic mediated contractile response and 2) an unaltered sensitivity (EC_{50}) to alpha-adrenergic agonists. Results from these studies will provide potential mechanisms responsible for the severely diminished diaphragmatic hyperemic response with contractions after prolonged MV as well as pathways for intervention to address problematic weaning.

Chapter 3 - Methods

Animals

Female Sprague-Dawley rats (4-8 month old, ~350 g) were obtained from Charles River Laboratories (Boston, MA, USA) for this investigation. Data were collected from either spontaneously breathing (SB, n = 9) animals or those successfully subjected (i.e., maintained arterial PO_2, PCO_2 and pH within normal ranges throughout the protocol, as described below) to prolonged (6 h) mechanical ventilation (MV, n = 6). In each animal, two first-order (1A) arterioles from the medial costal diaphragm were used for the isolated vessel experiments, and the respective responses were averaged. The Sprague-Dawley rat was chosen due to the similar properties (e.g. anatomical and physiological) of its diaphragm to the human diaphragm (52, 54, 66). 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*.

Mechanical Ventilation

All surgical procedures were performed using aseptic techniques. Animals in the MV groups were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), tracheostomized and connected to a volume-cycled ventilator (Kent Scientific PhysioSuite; Torrington, CT). A catheter was implanted in the carotid artery for measurement of blood pressure and periodic blood sampling (every 3 h) for analyses of blood gases (GemPremier 3000, Instrumentation Laboratory, Bedford, MA). Arterial O_2 saturation was monitored continuously by using a Mouse OX (Ken Scientific, Torrington, CT) placed around the rat's foot. Expired PCO_2 was measured

continuously using a microcapnograph (Kent Scientific CapnoScan, Torrington, CT) and maintained at ≤ 30 mmHg to minimize carotid chemoreflex stimuli and render the diaphragm inactive. Arterial PO_2 and pH were maintained within the normal range (70 to 110 mmHg, and 7.35 to 7.45, respectively) by minor adjustments to minute volume. A catheter was placed in the jugular vein for infusion of sodium pentobarbital (10 mg/kg/hr) and fluids, when necessary. Body temperature was monitored (via rectal thermometer) and maintained at 37 ± 1°C by use of a recirculating heating blanket. Body fluid homeostasis was maintained by administering an electrolyte solution (i.v., ~2 mL/kg/hr; 0.9% saline 5.0 pH, Na^+ [154mM], Cl^- [154 mM]). Operative care during the MV period included expressing the bladder, removal of airway mucus, lubricating the eyes and rotating the animal. The ventilator was maintained at an average breathing frequency of 70 ± 10 breaths/min and tidal volume of 6-7 mL/kg of body weight.

Isolated Microvessel Technique

To determine vasomotor control, resistance arterioles (<200 μm intraluminal diameter) were isolated from the medial costal diaphragm and studied *in vitro* to remove potentially confounding metabolic, humoral, and neural influences. Resistance arterioles were harvested from diaphragms of spontaneously breathing or prolonged MV animals. With the aid of a dissecting microscope (Olympus SVH10), first-order (1A) arterioles from the medial costal diaphragm muscle were isolated and removed from the surrounding muscle tissue as previously described (1). The arterioles (length, 0.5–1.0 mm; inner diameter, 90–175 μm) were transferred to a Poly(methyl methacrylate) chamber containing albumin-supplemented physiological saline solution (PSS) equilibrated with room air. Each end of the arteriole was cannulated with glass micropipettes and secured with nylon suture (11-0, ophthalmic suture, Alcon Laboratories, INC, Texas). Following cannulation, the microvessel chamber was transferred to the stage of an

inverted microscope (Olympus IX70) equipped with a video camera (Panasonic BP310), video caliper (Colorado Video 307A) and data-acquisition system (PowerLab) for on-line recording of intraluminal diameter. Arterioles were initially pressurized to 90 cmH_2O with two independent hydrostatic pressure reservoirs. Leaks were detected by pressurizing the vessel, and then closing the valves to the reservoirs and verifying that intraluminal diameter remained constant. Arterioles that exhibit leaks were discarded. Arterioles that were free from leaks were warmed to 37 °C and allowed to develop initial spontaneous tone during a 30–60 min equilibration period. Alpha-adrenergic responses (via norepinephrine (NE) and phenylephrine (PE)) and the passive-pressure responses were assessed in the isolated vessels as described below.

Alpha-adrenergic responses

Vessels used for alpha-adrenergic dose responses were equilibrated in PSS at 37 °C at 90 cmH_2O for 60 minutes and rinsed every 20 minutes with PSS. Thereafter, contractile responses to cumulative doses (10^{-9} to 10^{-4} M) at 3 minute intervals of norepinephrine (NE) and phenylephrine (PE) were determined and intraluminal diameter measured at the end of each 3 minute interval.

After completion of the final concentration-response or pressure-response, vessels were incubated with warm solution (pH = 7.4), free of Ca^{2+}, for 60 minutes and were rinsed every 20 minutes with calcium-free PSS. Following a 60 minute normalization period, vessels were exposed to one 80 μl (10^{-4}M) dose of SNP and maximal diameters were recorded.

Data Analysis

Maximal diameter and body weight were analyzed with one-way ANOVA. Vasomotor responses were evaluated by Two-way ANOVA to detect main effects between (SB and MV) or within groups. Post-hoc analyses were performed using a Tukey's test. All data are presented as means ± SE. Significance was established at $P \leq 0.05$.

Spontaneous tone development and responses to norepinephrine and phenylephrine were expressed as the percent constriction relative to maximal diameter and was calculated using the formula below (57):

$$\text{Spontaneous tone } (\%) = [(ID_{max} - ID_b) / ID_{max}] \times 100$$

where ID_{max} represents the maximal inner diameter, and ID_b represents the baseline inner diameter following the 60 minute equilibration period.

Vasoconstrictor responses to NE and PE were expressed as the percentage change from baseline diameter based on the formula (57):

$$\text{Constriction } (\%) = [(ID_b - ID_s) / ID_b] \times 100$$

where ID_b is the initial baseline diameter recorded immediately prior to the vasoconstrictor agonist addition, and ID_s represents the steady-state diameter recorded following each dose of the drug.

Chapter 4 - Results

Prolonged MV reduced α_1 –mediated vasoconstriction and α-adrenergic sensitivity

Diaphragm mass (g), spontaneous tone (%), maximal diameter (μm), wall thickness (μm), and wall-to-lumen ratio (μm) were not different between groups (Table 1). Vasoconstrictor responses to NE were not different between SB and MV (Figure 4.1A; Table 1). However, with PE, maximal constriction (max %) was diminished by 20% (Figure 4.1B; Table 1) in vessels from the MV group (SB, 37.3 ± 6.7% vs. MV, 19.0 ± 1.9%; $p \leq 0.05$). Compared to SB, sensitivity (EC_{50}) was altered to both NE and PE with prolonged MV ($p \leq 0.05$; Table 1); following 6 h MV, diaphragm arterioles required a higher concentration of NE and PE to elicit the same response exhibited from the SB group (Figure 4.2A and 4.2B).

Table 1. Spontaneous tone development, vessel characteristics, and maximal responses of diaphragm arterioles from spontaneous breathing (SB) and prolonged MV (6 h MV) groups.

Measurements	SB	PMV
	(n = 9)	*(n = 6)*
Absolute mass		
Body (g)	390 ± 6.1	302 ± 14.3
Diaphragm (g)	1.4 ± 0.1	1.2 ± 0.7
Vessel Characteristics		
Spontaneous Tone, %	12.6 ± 3.5	5.9 ± 1.3
Maximal Diameter, µm	184 ± 11	186 ± 12
Wall Thickness, µm	26 ± 2	28 ± 2
Wall-to-Lumen ratio, %	7.1 ± 2.4	6.7 ± 1.9
Maximal Responses		
NE, Max %	33.2 ± 5.9	26.1 ± 3.4
PE, Max %	37.3 ± 6.7	19.0 ± 1.9 *
EC50 (x 10^{-7} M)		
NE	5.25 ± 2.0	43.0 ± 18.0 *
PE	23.0 ± 7.2	93.0 ± 11.1 *

Values are means ± SE. *n* = vessels studied *in vitro*. *NE; norepinephrine, PE; phenylephrine.*

* Significant ($p \leq 0.05$) difference vs. SB

Figure 4.1. (A) Dose responses to non-selective α-adrenergic agonist, norepinephrine (NE) and (B) Dose responses to α_1-specific agonist, phenylephrine (PE) during SB (n = 9) and 6 h MV (n = 6). *P≤0.05 versus SB.

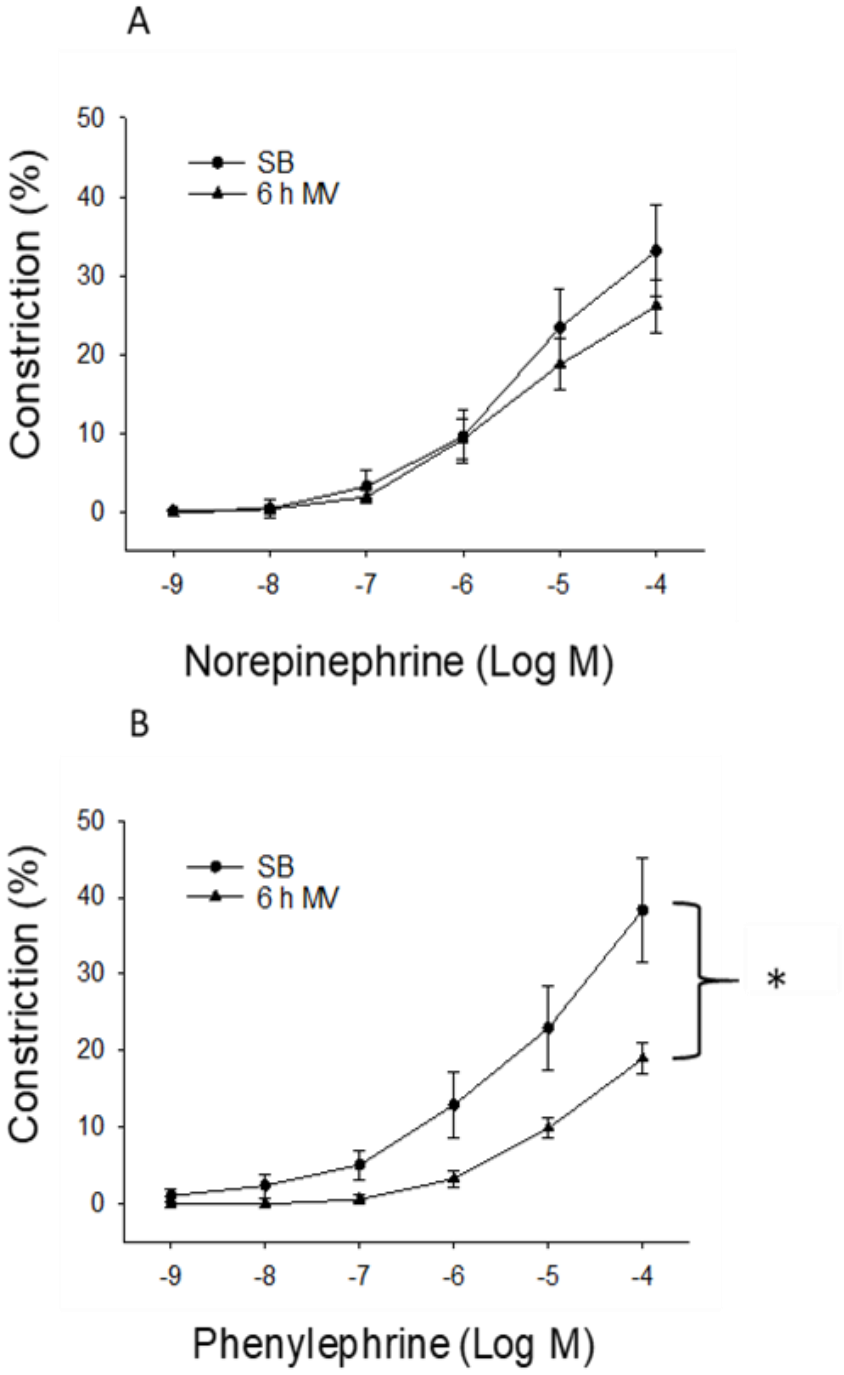
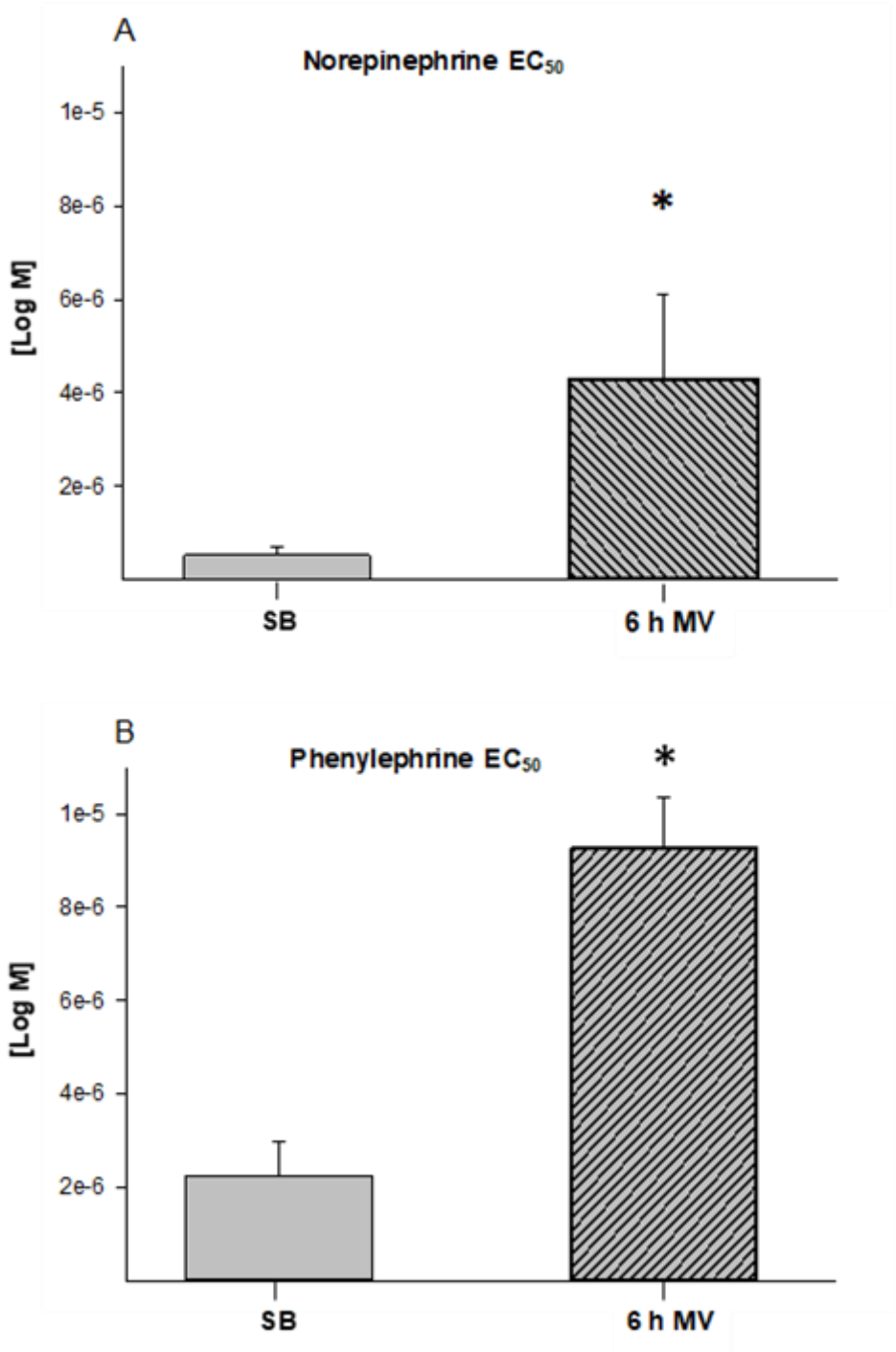


Figure 4.2. EC_{50}, the concentration of a drug that elicits half-maximal response, of (A) norepinephrine (NE) and (B) phenylephrine (PE) during SB (n = 9) and 6 h MV (n = 6).*P≤0.05 versus SB.



Chapter 5 - Discussion

Altered alpha-adrenergic responses

Alpha-adrenergic receptors (α_1- and α_2-receptors) are expressed in resistance vasculature and mediate vasoconstriction by inducing vascular smooth muscle contraction. We examined α_1 and α_2-adrenergic receptor contributions to vasoconstriction in diaphragm first-order arterioles (1A) via norepinephrine (NE) and phenylephrine (PE), a non-selective α-adrenergic agonist and α_1-specific agonist, respectively. After prolonged MV, diaphragm arterioles showed no difference in vasoconstriction to NE compared to the SB group, yet, contrary to our hypothesis, displayed a 20% reduction in vasoconstriction in response to PE (Figure 4.1B), suggesting a possible reduction in α_1-mediated vascular control. Albeit the exact mechanism for the reduced α_1-mediated vasoconstriction produced in this study was not determined, prior evidence suggests that the location and sensitivity of the adrenergic receptors modulate vascular tone (1, 55). Aaker and Laughlin (1), found that second-order arterioles (2A) in the diaphragm are primarily regulated via α_1-receptors, with little contribution from α_2-receptors, which is similar to those from the cremaster muscle (60). The diaphragm has an enormous capacity for flow (65), similar to other highly oxidative skeletal muscles (4) but dissimilar in that it is never truly quiescent in health. Further, the diaphragm demonstrates a specialized microcirculation (e.g., countercurrent capillary flow (37)); structurally and functionally optimized to facilitate oxygen transfer (66). However, the diaphragm is particularly susceptible to ischemia-reperfusion damage and associated downward shifts in the force-frequency relation (89). Therefore, it could be hypothesized that the diaphragm demonstrates a blunted vasoconstriction in health to neural/humoral stimuli versus other skeletal muscle to maintain O_2 delivery. The literature is relatively scant on this topic. However, Supinski et al. (90) demonstrated norepinephrine

infusion increases blood flow to the diaphragm in dogs. Ward and Hussain (100), showed that α_1-AR activation (via phenylephrine infusion) in the diaphragm of dogs increased O_2 extraction, similarly to the increased O_2 extraction in the diaphragm of rats with 6 h of MV (16). Further, contrary to the robust constriction to these agonists in arterioles of skeletal muscle (42, 43, 81), diaphragm arterioles are less responsive to α-adrenergic constriction; which may be beneficial in maintaining diaphragmatic blood flow when sympathetic nerve activity and plasma NE concentrations are elevated (i.e. exercise) (1). The blunted α_1-adrenergic and unaltered α_2-adrenergic vasoconstriction following MV may stem from downstream G-protein coupling, second-messenger signaling (i.e. inositol 1, 4, 5-triphosphate (IP3)), and/or intracellular Ca^{2+} release. Since α_1- and α_2- mediated smooth muscle contraction work via different downstream G-protein coupling, and second messenger signaling (103, 14) this could explain, in part, the unaltered α_2 -response with a reduced sensitivity to both agonists.

Ramifications of rapid vasomotor dysfunction in the diaphragm with MV

In order to sustain diaphragmatic metabolic and contractile function, there must be a tight coupling of $\dot{Q}o_2$ to myocyte energetics and $\dot{V}o_2$ (63, 66). During weaning from MV, there is five-fold increase in diaphragm venous blood lactate, a ~30% decrease in diaphragm glycogen stores, and 50% reduction in [ATP] and [CP] (45): all of which indicate an inability to match $\dot{Q}o_2$ to $\dot{V}o_2$, similar to the diminished microvascular oxygenation as previously observed (16). During severe shock states, when $\dot{Q}o_2$ is markedly reduced, the activation of diaphragm α-ARs via catecholamines, may accelerate the development of an $\dot{Q}o_2$ limitation and hinder the ability to sustain ventilation (100). Further, with prolonged MV, there is an increase in vascular resistance (16), and the reduced sensitivity to α-adrenergic agonists demonstrated herein, suggesting there is a prolonged vasoconstriction of diaphragm vasculature during MV. Therefore, prolonged α-

adrenergic activation *in vivo* appears to be contributing to the reductions in $\dot{Q}_{O_2}$ during MV and exacerbates the inability of the diaphragm to augment blood flow in response to contractions following MV.

Limitations

The SB group was not subject to the same duration of anesthesia; however, if this was an anesthesia issue it would have to be selective to the diaphragm, as other skeletal muscle does not demonstrate reductions in blood flow over the 6 h period during anesthesia (16). No data that we are aware of demonstrates the diaphragm to be a selective target of systemic anesthesia. Our investigations were in the absence of *in vivo* neural and humoral influences, which is common for all isolated vessel preparations, and allows investigation of specific mechanisms of dysfunction. Diaphragm quiescence was not verified directly, however, the diaphragm is inactive in anesthetized rats during prolonged controlled MV (70, 16) and has been verified through electromyography (80). As PCO_2 was maintained $\leq$ 30 mmHg, there was likely no sensory input to the respiratory control center by carotid chemoreceptor activation. In addition, pleural pressures were not determined during MV and could have affected diaphragmatic blood flow. However, previous work demonstrates that hyperinflation of the lungs fails to significantly alter diaphragmatic blood flow (36).

Conclusion

Following 6 hours of MV, altered α-adrenergic responses of diaphragm arterioles occur through both endothelium-dependent and –independent pathways. This is the first study, to our knowledge, demonstrating that diaphragm arteriolar dysfunction is associated with prolonged MV and likely contributes to ventilator-induced diaphragm dysfunction (VIDD) and weaning difficulties. Further studies are needed to investigate the possible role of other vasoactive

mediators such as Angiotensin-II and Endothelin-1, which may be upregulated with MV, as well as the potential contribution of oxidative stress and crosslinking extracellular matrix proteins to vasomotor alterations with prolonged MV.

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