

Skeletal and respiratory muscle oxygen transport in pulmonary hypertension

by

Kiana Marie Schulze

B.S., Kansas State University, 2019

M.S., Kansas State University, 2020

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Kinesiology
College of Health and Human Sciences

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2025

Abstract

Pulmonary hypertension (PH) is a disease characterized by pulmonary vascular remodeling and elevated pulmonary arterial pressures which subsequently impairs gas exchange in the lungs and eventually, right ventricular function. Hallmarks of PH include dyspnea and exercise intolerance which, despite previous notions, is not solely attributable to cardiopulmonary dysfunction. In these investigations, we sought to determine whether skeletal and respiratory muscle blood flow and vascular function are impaired in PH at rest and during contractions or exercise. Specifically, we used intravital microscopy, fluorescent microspheres, and pressure myography to explore these questions in a monocrotaline rat model of PH. Our findings demonstrate that resting and exercising blood flow and oxygen transport are lower in skeletal muscle via convective and diffusive impairments in PH compared with healthy controls. In respiratory muscle (i.e., the diaphragm), PH rats display a regional redistribution toward the ventral and crural regions of the diaphragm during contractions and submaximal exercise. These regions are not expected to contribute as greatly to ventilatory work and this observation therefore unveils a mechanism potentially underlying diaphragmatic weakness and failure in PH. Our findings suggest that the medial costal diaphragm arterioles are less reactive, both to vasodilatory stimuli as well as changes in intraluminal pressure, which may help explain the basis for this regional redistribution. Finally, we've found that there is a heightened diaphragm blood flow during submaximal exercise in PH compared with healthy animals that is concurrent with a lower skeletal muscle blood flow. This redistribution of blood flow toward the diaphragm and away from working muscle during exercise is likely related to diaphragm vascular dysfunction and exercise intolerance. As such, these derangements may provide a specific target for therapeutic interventions that could potentially normalize muscle blood flow distribution to that of healthy individuals and help ameliorate exercise intolerance. This body of work provides novel insights into skeletal and respiratory muscle oxygen delivery alterations at rest and during exercise in PH, providing evidence for oxygen transport-related mechanisms that may predicate both dyspnea and exercise intolerance in PH. Future directions will hopefully assess targeted therapeutic strategies aimed at improving diaphragm vascular function with the goal of restoring blood flow and oxygen delivery, improving O₂ delivery-to-O₂ demand matching and improving the quality of life and prognosis in PH.

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Acknowledgements

I would first like to acknowledge my doctoral committee: Drs. David C. Poole, Timothy I. Musch, Bradley J. Behnke, Judy Muller-Delp, and Stephanie Hall. As my mentors, you've trained me as a scientist, but more importantly, you've shaped who I am. Fortunately, you are a remarkably genuine, resilient, enthusiastic, and dedicated group of individuals. It's truly been an honor to learn from you for the past 5 years. Thank you for fostering curiosity, collegiality, and discovery, and for always making time to share stories and jokes in the corridor or at the pub. I will dearly miss our daily conversations, but I treasure our friendships that will continue regardless of our proximity.

I would also like to thank the graduate students, faculty, and staff with whom I shared time and memories at Kansas State, I feel privileged to have experienced this alongside you and am so grateful for our lasting friendships. Ramona Weber, my #RatLab mate from start to finish, I could not have completed this without you, whether it was lending a helping hand with data collection, making hype playlists and silly memes, or just being a supportive friend. I cherish our shenanigans in Coles Hall, and I am sure we will shenan-again as life takes us to new places. Above all, I appreciate each of you for making my time in graduate school so much fun. They really mean it when they say college is the best 9.5 years of your life, and I have you all to thank for that.

I owe a special thank you to my family. Thank you for always believing in me and instilling in me kindness, wisdom, and a sense of humor. Without your unwavering support, this would not have been possible. Finally, Andrew Horn, thank you for encouraging, challenging, and inspiring me. You make me better every day.

Preface

Chapters 2-5 of the present dissertation represent peer-reviewed articles that have been published in scientific journals and the necessary permissions have been obtained to publish them in this dissertation. These chapters are written in published format and the citations are listed below.

Schulze, K.M., Weber, R.E., Horn, A.G., Colburn, T.D., Ade, C.J., Poole, D.C. and Musch, T.I., 2022. Effects of pulmonary hypertension on microcirculatory hemodynamics in rat skeletal muscle. *Microvascular research*, 141, p.104334.

Schulze, K.M., Horn, A.G., Weber, R.E., Behnke, B.J., Poole, D.C. and Musch, T.I., 2023. Pulmonary hypertension alters blood flow distribution and impairs the hyperemic response in the rat diaphragm. *Frontiers in Physiology*, 14, p.1281715.

Schulze, K.M., Horn, A.G., Muller-Delp, J.M., White, Z.J., Hall, S.E., Medarev, S.L., Weber, R.E., Poole, D.C., Musch, T.I. and Behnke, B.J., 2024. Pulmonary hypertension impairs vasomotor function in rat diaphragm arterioles. *Microvascular Research*, 154, p.104686.

Schulze, K.M., Weber, R.E., Horn, A.G., Hageman, K.S., Kenney, N.J., Behnke, B.J., Poole, D.C. and Musch, T.I., 2024. Skeletal and respiratory muscle blood flow redistribution during submaximal exercise in pulmonary hypertensive rats. *The Journal of Physiology*.

Chapter 1 - Introduction

O₂ Transport Cascade

Oxygen (O₂), the “food of life” [Sendivogius, 1604] governs the survival and function of every tissue in the body. In resting humans, ~100 quintillion (10¹⁸) molecules of O₂ are transported every second via convective and diffusive mechanisms from the atmosphere to tissue, where it is utilized for adenosine triphosphate (ATP) synthesis in the mitochondrial reticulum [Poole et al., 2022].

With each breath, the negative pressure generated by contraction of the diaphragm facilitates convective transfer of air into the lungs. At sea level, the partial pressure of oxygen (PO₂) is ~159 Torr (20.93/100 * 760). That air must be warmed and moistened (vapor pressure = 47 → 149 Torr), and by the time it enters the alveolus, the sacs within the lung that fill with air, providing a surface area the size of a tennis court for gas exchange into the pulmonary capillaries, PO₂ is ~100 Torr due to the mixing of gases within the lung. Fick’s Law of Diffusion dictates the flux of oxygen from the alveolus into the blood:

$$\dot{V}O_2 = A/T \times D \times \Delta P$$

where A is area, T is thickness, D is diffusing capacity, and ΔP is the pressure gradient [West, 2012]. Alveolar-blood ΔP is 60 mmHg, so oxygen diffuses across the thin walls of the alveolus and the capillaries into the blood.

Oxygenated blood is transported via the arterial tree, beginning with large arteries such as the aorta, to the capillary bed where oxygen is offloaded to the tissue. The arterioles (resistance vasculature) are primarily responsible for regulating the distribution of blood flow. Large arteries contain more elastin, while smaller arterioles contain more smooth muscle which allows them to contract (vasoconstrict) or relax (vasodilate). The endothelial layer is the innermost

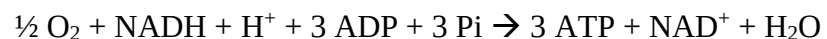
layer and is sensitive to mechanical stimuli such as shear stress from blood flow, and acts as a sensor and producer of vasoactive mediators.

Flux of O₂ into the myocyte is determined by both the pressure gradient and the surface area for oxygen exchange (DmO₂) [Federspiel & Popel, 1986; Groebe & Thews, 1990].

$$\dot{V}O_2 = DmO_2 \times (P_{mv}O_2 - P_{im}O_2).$$

In the capillary bed of the tissue, specifically muscle tissue, the capillary or microvascular PO₂ (P_{mv}O₂) is ~35 mmHg [Hirai et al., 2018] while the intramyocyte PO₂ (P_{im}O₂) is ~10 mmHg [Gorczyński & Duling, 1978]. This pressure gradient facilitates oxygen diffusion across the carrier-free interstitial space into the myocyte. In health, ~80% of capillaries support red blood cell (RBC) flux, but in states of disease, increased venous pressures, impaired vasodilation/heightened constriction, exaggerated sympathetic nerve activity, etc. may cause stopped or intermittent flow, such that the surface area of RBCs adjacent to the myocyte is reduced in the absence of substantial increases in capillary hematocrit [Kindig et al., 1999; 2002].

Once in the myocyte, oxygen transport is facilitated by myoglobin to the mitochondrial reticulum, an interconnected matrix where ATP synthesis takes place via cellular respiration [Glancy et al., 2015]. This ATP serves as the energy source necessary for processes involved in muscular contraction. O₂ acts as the final electron acceptor in the electron transport chain and, as such, is necessary for ATP generation via oxidative phosphorylation which can be illustrated using the P/O ratio:



Cardiovascular Response to Exercise

At the onset of dynamic exercise, ATP demand increases in a square-wave fashion while oxygen uptake increases exponentially to steady-state. In the initial minute of exercise, or when energetic demands are greater than what is supplied by oxidative phosphorylation, non-aerobic sources supply the additional ATP needed, which results in lactate buildup and metabolic acidosis as the byproducts of glycolysis accumulate more quickly than they can be removed. As exercise intensity increases, oxygen consumption increases linearly with workload to maximal O₂ uptake ($\dot{V}O_{2\max}$) [Hill & Lupton, 1923]. $\dot{V}O_{2\max}$ is determined by the Fick equation:

$$\dot{V}O_2 = \dot{Q} \times (CaO_2 - CvO_2)$$

where $\dot{Q}$ is cardiac output (the product of heart rate and stroke volume) and CaO_2 and CvO_2 are arterial and venous oxygen contents, respectively. CaO_2 and CvO_2 are determined by the pressure of oxygen, the concentration of hemoglobin molecules, and the saturation of those hemoglobin molecules in arterial or mixed venous blood, respectively.

In response to exercise, stroke volume and heart rate increase, increasing blood flow throughout the body. Blood flow is redistributed toward working skeletal muscle and away from the viscera to maintain mean arterial pressure and support the energetic demands of contracting muscle. Functional hyperemia to skeletal muscle is mediated via chemical and mechanical signals. The initial vasodilatory response occurs within 1 second of contraction onset, which is followed by a sustained phase of hyperemia that is mediated by a combination of metabolite production, sympathetic neural input, and the muscle pump [Delp & Laughlin, 1998]. Contraction-induced hyperemia increases capillary hematocrit, RBC flux and RBC velocity in

the capillary bed [Kindig et al., 2002]. Thus, CvO_2 is lowered due to increased offloading of O_2 to the working muscle, while CaO_2 remains relatively constant.

Exercise requires complex and coordinated responses of multiple bodily systems to facilitate oxygen transport and maintenance of cellular functions despite alterations in pH, temperature, and ATP demand. In disease states (i.e., chronic heart failure, emphysema, diabetes), one or several steps of the O_2 cascade may be compromised, impairing exercise capacity [Poole et al., 2012]. These aberrations may be central (impaired pulmonary gas exchange, inadequate increases in cardiac output) and/or peripheral (blunted vascular reactivity, capillary hemodynamic alterations, mitochondrial dysfunction). The extent to which the peripheral steps of the O_2 transport cascade are affected in PH is not fully understood.

Pulmonary Hypertension

Pulmonary hypertension (PH) is characterized by pulmonary vascular remodeling in the form of vascular smooth muscle and endothelial cell proliferation and hypertrophy, adventitial stiffening, and consequent increases in pulmonary arterial pressures (PAP) >20 mmHg [Simonneau & Hoeper, 2019]. There are 5 groups of PH, the first of which is pulmonary arterial hypertension (PAH), which is distinct from the other groups because it develops independent of other pathologies or thromboembolism. All etiologies of PH present the risk of right ventricular (RV) hypertrophy and eventual failure. PH was first described in 1891 [von Romberg], but it wasn't until 60 years later when David T. Dresdale, a trainee of Andre F. Cournand and Dickinson W. Richards who are credited for implementing right atrial catheterization, coined the term “primary pulmonary hypertension” (now also regarded as PAH) in print [Dresdale et al., 1951]. Prior to this, it was referred to as “cor pulmonale” or using other nomenclature [McGinn & White, 1935], which made it difficult to differentiate between PAH, and PH or right

ventricular (RV) hypertrophy associated with various pathologies. The naming of this disease allowed for further investigation into diagnostic methods and the mechanisms specific to the pathogenesis and symptoms of PAH. In this dissertation, PH and PAH will be used interchangeably, and if another etiology of PH is discussed, it will be identified accordingly.

Central Cardiopulmonary Dysfunction

In PAH, the initial stimulus for pulmonary vascular remodeling and the sequence of pathogenesis is not fully understood. The principal hypothesis which has emerged is that inflammatory endothelial injury due to mechanical or chemical factors promotes migration of mast cells, lymphocytes, leukocytes, and macrophages to the site of injury [Tuder, 2017]. Aberrant signaling related to growth factors, matrix metalloproteinases, immune cells, and chemokines consequently leads to cellular apoptosis, phenotypic alterations, proliferation of apoptosis-resistant cells, and collagen deposition in the vascular wall, resulting in the characteristic remodeling, stiffening, and vascular lesions [Chelladurai et al., 2012; Guignabert & Dorfmuller, 2017; Voelkel et al., 2012]. Thus, a vicious cycle of inflammation, oxidative stress, imbalances in vasoactive mediators, vascular remodeling, and increased vascular resistance and pulmonary pressures ensues, giving rise to ventilation-perfusion mismatch and impaired pulmonary gas exchange [Bello-Klein et al., 2018; Christman et al., 1992; Dorfmuller et al., 2003; Giaid et al., 1993; Giaid & Saleh, 1995; Giaid, 1998; Guignabert & Dorfmuller, 2017].

For many years, PH was regarded as an “orphan disease” which classifies a group of diseases that are considered rare and, as such, have few to no treatment options [Voelkel et al., 2012]. Now, several treatments are available, primarily focusing on pulmonary vasodilation (i.e., prostacyclin infusion, endothelin receptor antagonists, calcium channel blockers,

phosphodiesterase 5 inhibitors, and soluble guanylyl cyclase stimulators) [Benza et al., 2024; Thomas et al., 2020]. However, it remains unknown how to reverse the pathogenesis in PH patients. Furthermore, therapeutics aimed at the pulmonary circulation may have negative effects on the heart, which, given that RV ejection fraction is the strongest predictor of survival [van de Veerdonk et al., 2011]. could exacerbate disease progression. The RV is crescent shaped and thin-walled compared to the left ventricle and is not intended to withstand prolonged increases in pressure. Elevated pulmonary vascular resistance and PAP increase RV afterload, triggering a compensatory hypertrophy followed by dilated cardiomyopathy. Increased workload of the RV in combination with structural remodeling results in O₂ demand-delivery imbalance, ischemia, and intrinsic cardiomyocyte alterations which are all suspected to play a role in the eventual RV failure with PH [Bogaard et al., 2009].

Exercise intolerance and dyspnea are characteristic of PH and were regarded as consequences of impaired gas exchange, heightened pulmonary vascular pressures, and RV dysfunction for much of the 20th century. Increased $\dot{V}_e/\dot{V}_{CO_2}$ in PH patients at rest and during exercise supports ventilatory insufficiency in some regard, and patients often exhibit low resting PaCO₂, which suggests chronic hyperventilation that may predispose respiratory muscles to fatigue [Sun et al., 2001]. Work within the last 2 decades has focused on skeletal and respiratory muscle-specific dysfunction, beyond the limitations imposed by central cardiopulmonary impairments, and how this contributes to PH-induced dyspnea and exercise intolerance.

Respiratory Muscle Dysfunction

Respiratory muscle weakness was first characterized in PH in 2005 [Meyer et al.] when it was reported that male and female PH patients display lower maximal inspiratory and expiratory pressures compared with healthy controls. In 2007, another study corroborated these results and

supplemented this with findings that both volitional and non-volitional pressures were lower in PH patients [Kabitz et al., 2007]. Furthermore, this weakness was associated with 6-minute walking distance (6MWD) in patients, suggesting for the first time that diaphragmatic weakness may play a role in exercise intolerance in PH. It was later demonstrated that diaphragm contractile impairments in PH patients occurred concomitant with diaphragm fiber atrophy. This was supported in the same study by findings of lower diaphragm fiber force generation, reduced cross sectional area of both fast and slow twitch fibers, and increased expression of proteolytic markers (E3-ligases MAFbx and MuRF-1) in monocrotaline-induced PH rats compared with controls, providing for the first time more mechanistic answers to the underlying cause of respiratory muscle weakness in PH [de Man et al., 2011]. However, the literature is not consistent as it regards fiber atrophy and markers of proteasomal degradation in the PH diaphragm. Fiber atrophy has been demonstrated in PH patients, monocrotaline-PH rats [de Man et al., 2011], and mice [Ahn et al., 2013], but was not seen in the Sugren-hypoxia rat model of PH [Kosmas et al., 2022]. This atrophy has been attributed to increased ubiquitin proteasomal degradation in some [de Man et al., 2011] but not all [Ahn et al., 2013; Kosmas et al., 2022] reports, suggesting muscle wasting may not be the only culprit for weakness and contractile impairments. Diaphragm tissue from de Man et al. [2011] was also used to determine that reduced contractile function may be related to sarcomeric alterations [Manders et al., 2012]. Reduced calcium sensitivity and force generation per cross bridge in PH diaphragm fast-twitch fibers indicate that impairments at the level of the sarcomere are present in fast twitch fibers in the PH diaphragm. Additionally, it is worth noting that previous investigations have demonstrated increased calcium accumulation in diaphragm strips from PH rats, which could lead to the reduced sensitivity over time [Kanj et al., 1999]. Furthermore, it has been suggested

that there is a fiber-type “shift” in the diaphragm that may be due to increased FoxO1 which could inhibit Type 1 fiber differentiation, increasing the T2/T1 fiber ratio, sowing the seeds for diaphragmatic fatigue [Kosmas et al., 2022].

Other mechanisms suggested to play a role in diaphragm contractile impairments and weakness are mitochondrial dysfunction, oxidative stress, and inflammation. Markers of mitochondrial function and biogenesis are lower in PH diaphragm tissue (e.g., ↓ PCG-1 α , Tfam, and Sirt-1) and genomic data reveals downregulation of genes associated with mitochondrial function and an upregulation in genes associated with inflammation [Kosmas et al., 2022]. Oxidative stress may also be tied to mitochondrial function; however, two investigations have seen no change in protein oxidation or markers of oxidative stress in diaphragm tissue from animals with severe PH [Ahn et al., 2013; Manders et al., 2012]. Conversely, administration of a SOD/catalase mimetic following monocrotaline administration prevented reductions in diaphragm bundle force generation, so there may be an initial role of oxidative stress in PH diaphragm pathogenesis [Himori et al., 2017]. Importantly, all of these studies, to the best of our knowledge, utilized a right ventricular failure animal model of PH. Although hypoperfusion is often suggested as a potential mechanism, none have investigated the potential role of altered diaphragm blood flow in mediating the contractile dysfunction and muscle weakness in PH.

Peripheral Skeletal Muscle Dysfunction

Some of the investigations previously discussed suggest that contractile impairments in PH are diaphragm-specific [de Man et al., 2011; Manders et al., 2012]; however, others note similar impairments in skeletal muscle [Kosmas et al., 2022]. In fact, Bauer et al. [2007] demonstrated correlations between grip strength, 6MWD, and inspiratory and expiratory strength in PH patients. This was the first study to suggest a “generalized myopathy” in PH. To date, several

studies postulate that exercise intolerance and impaired muscle strength in PH patients [Barbosa et al., 2011; Batt et al., 2014; Breda et al., 2014; Mainguy et al., 2010; Malenfant et al., 2015a; Malenfant et al., 2015b; Potus et al., 2014] and animal models [Kosmas et al., 2022; Long et al., 2022] are related to a lower muscle oxygen delivery. Although PH patients present with reduced blood oxygen saturation and a diminished stroke volume response to exercise [Holverda et al., 2006; Sun et al., 2001], decreased skeletal muscle strength and/or exercise capacity occurs independent of central hemodynamic measurements (PAP, cardiac output, cardiac index) [Bauer et al., 2007; Breda et al., 2014; Dimopoulos et al., 2013; Mainguy et al., 2010], indicating the presence of vascular dysfunction and intrinsic muscle alterations beyond the cardiopulmonary system.

Intrinsic skeletal muscle abnormalities include muscle fiber atrophy (in some [Batt et al., 2014] but not all [Breda et al., 2014; Kosmas et al., 2022; Malenfant et al., 2015b; Potus et al., 2014]), aberrant mitochondrial morphology [Malenfant et al., 2015a] and mitochondrial dysfunction [Enache et al., 2013; Kosmas et al., 2022; Malenfant et al., 2015a], increased type 2:type 1 fiber ratio (in some [Batt et al., 2014; Breda et al., 2014; Kosmas et al., 2022; Mainguy et al., 2010; Malenfant et al., 2015a] but not all [Malenfant et al., 2015b; Potus et al., 2014]), greater reliance on glycolytic metabolism [Mainguy et al., 2010], lower citrate synthase activity [Enache et al., 2013; Malenfant et al., 2015a], and impaired force generation [Batt et al., 2014; Mainguy et al., 2010; Manders et al., 2015]. Moreover, endothelial dysfunction in large peripheral arteries (i.e., brachial) [Wolff et al., 2007], has been demonstrated in PH, along with reduced muscle oxygenation, greater fractional extraction, slower $\dot{V}O_2$ kinetics, decreased capillary density, impaired angiogenesis, longer reactive hyperemia time, and lower muscle blood flow [Barbosa et al., 2011; Dimopoulos et al., 2013; Long et al., 2022; Mainguy et al.,

2010; Malenfant et al., 2015b; Potus et al., 2014], further supporting the hypothesis that impaired muscle oxygen delivery may underlie contractile dysfunction and exercise intolerance.

Summary

Muscle function is determined by intricate matching of oxygen delivery to oxygen demand, which becomes crucial during metabolic stressors such as exercise where muscle O₂ requirements can increase 100-fold [Poole et al., 2011]. Pulmonary hypertension, traditionally regarded as a cardiorespiratory disease, also induces systemic dysfunction, specifically within respiratory and skeletal muscles. The purpose of this dissertation is to elucidate the role of oxygen transport perturbations in muscle dysfunction, providing insight into pathways to alleviate these symptoms which will enhance physical capacity and quality of life for individuals with PH.

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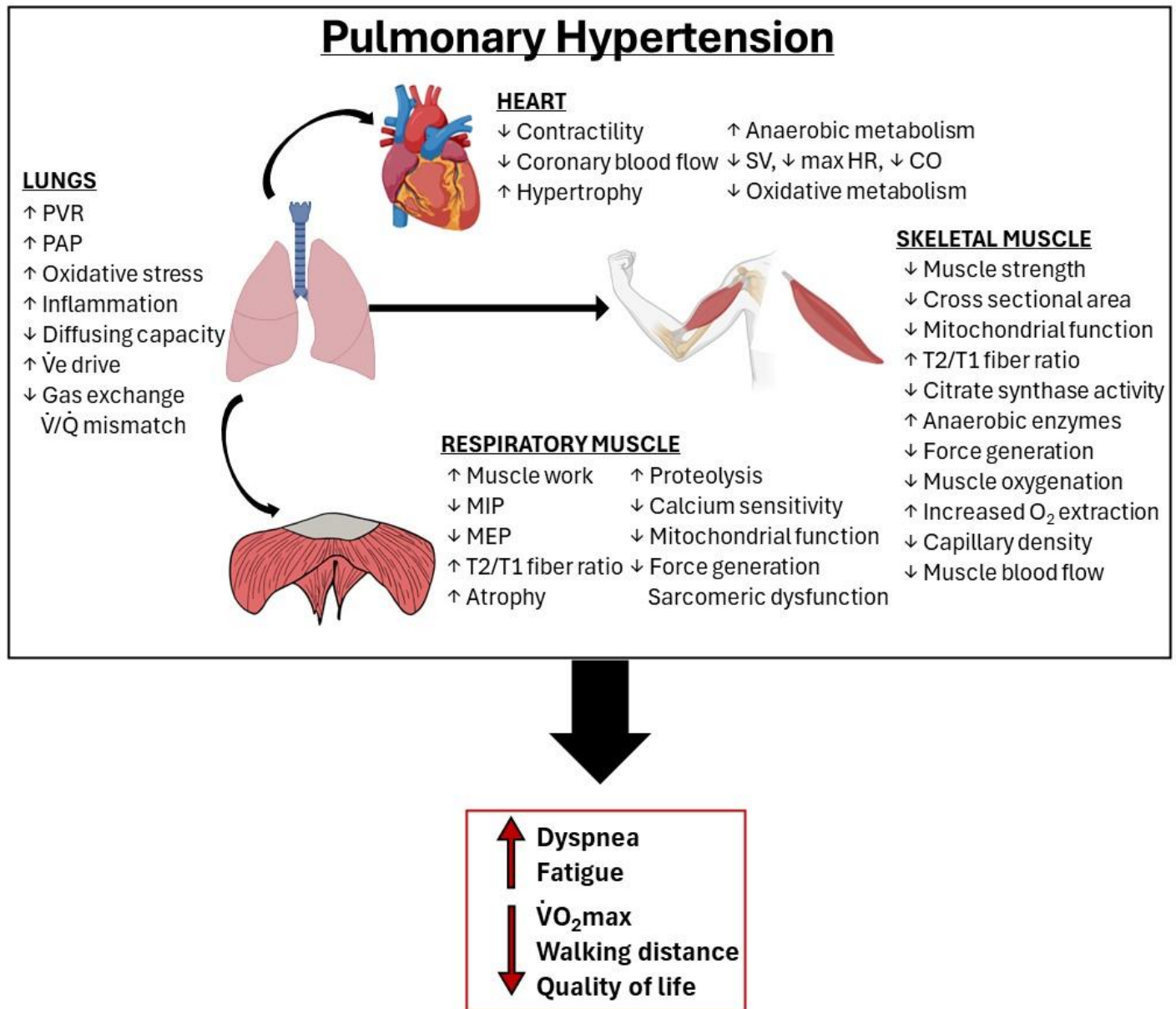
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Figure 1.1 Central and peripheral dysfunction in pulmonary hypertension.



Schematic representation of systemic dysfunction in pulmonary hypertension. PVR, Pulmonary vascular resistance; PAP, pulmonary arterial pressure; $\dot{V}_e$, ventilatory; $\dot{V}/\dot{Q}$, ventilation-perfusion; SV, stroke volume; HR, heart rate; CO, cardiac output; MIP, maximal inspiratory pressure; MEP, maximal expiratory pressure; T2/T1, type II/type I; $\dot{V}O_{2max}$, maximal oxygen uptake

Chapter 2 - Effects of Pulmonary Hypertension on Microcirculatory

Hemodynamics in Rat Skeletal Muscle

Abstract

Pulmonary hypertension (PH) has previously been characterized as a disease of the pulmonary vasculature that subsequently results in myocardial dysfunction. Heart failure compromises skeletal muscle microvascular function, which contributes to exercise intolerance. Therefore, we tested the hypothesis that such changes might be present in PH. Thus, we investigated skeletal muscle oxygen (O_2) transport in the rat model of PH to determine if O_2 delivery ($\dot{Q}O_2$) is impaired at the level of the microcirculation as evidenced via reduced red blood cell (RBC) flux, velocity, hematocrit, and percentage of capillaries flowing in quiescent muscle. Adult male Sprague-Dawley rats were randomized into healthy (n=9) and PH groups (n=9). Progressive PH was induced via a one-time intraperitoneal injection of monocrotaline (MCT; 50 mg/kg) and rats were monitored weekly via echocardiography. Intravital microscopy in the spinotrapezius muscle was performed when echocardiograms confirmed moderate PH (preceding right ventricular (RV) failure). At 25 ± 9 days post-MCT, PH rats displayed RV hypertrophy (RV/(Left ventricle+Septum): 0.28 ± 0.05 vs. 0.44 ± 0.11), pulmonary congestion, and increased right ventricular systolic pressure (21 ± 8 vs. 55 ± 14 mmHg) compared to healthy rats (all $P < 0.05$). Reduced capillary RBC velocity (403 ± 140 vs. 227 ± 84 $\mu\text{m/s}$; $P = 0.01$), RBC flux (33 ± 12 vs. 23 ± 5 RBCs/s; $P = 0.04$) and % of capillaries supporting continuous RBC flux at rest (79 ± 8 vs. $56 \pm 13\%$; $P = 0.01$) were evident in PH rats compared to healthy rats. When $\dot{Q}O_2$ within a given field of view was quantified (RBC flux x % of capillaries supporting continuous RBC flux), PH rats demonstrated lower overall $\dot{Q}O_2$ ($\downarrow 50\%$; $P = 0.002$). These data support that microcirculatory hemodynamic impairments ($\downarrow \dot{Q}O_2$ and therefore altered $\dot{Q}O_2$ -to- $\dot{V}O_2$ matching) may compromise blood-myocyte O_2 transport in PH. The mechanistic bases for decreased capillary RBC flux, velocity, and percentage of capillaries supporting RBC flow remains an important topic

Introduction

Pulmonary hypertension (PH) is a life-threatening disease characterized by exercise intolerance, dyspnea, and poor quality of life. Exercise intolerance is often defined as low maximal aerobic capacity ($\dot{V}O_{2\max}$), which is determined by both perfusive and diffusive oxygen (O_2) transport (Wagner et al., 1997). The capillary bed represents the functional and structural framework for substrate exchange in skeletal muscle. Any aberrations in capillary hemodynamics will impact perfusive and/or diffusive O_2 transport across the blood-myocyte interface, the former of which is determined primarily by red blood cell (RBC) flux (f_{RBC}) and O_2 content and the latter by the number of RBCs flowing in the capillaries or capillary bed adjacent to the myocytes at any instant. Elevated pulmonary vascular pressures in PH compromise pulmonary and cardiac function, often resulting in hypoxemia and attenuated cardiac output ($\dot{Q}_{TOT}$), which would effectively lower $\dot{V}O_{2\max}$ by reducing perfusive O_2 transport. Importantly, capillary density is correlated with reduced exercise capacity in PH (Mainguy et al., 2010), and may therefore impair blood-myocyte O_2 exchange at the level of the tissue, potentially highlighting a concomitant role for impaired diffusive transport to contribute to low muscle oxygenation seen in this disease state (Malenfant et al., 2015; Dimopoulos et al., 2013). Crucially, we are unaware of any direct in vivo analysis (via intravital microscopy) of skeletal muscle capillary hemodynamics in PH, which is critical for resolving the impact of this disease on capillary function and muscle O_2 delivery.

In left ventricular heart failure with reduced ejection (HFrEF), attenuations in $\dot{V}O_{2\max}$ result from impaired perfusion (blood flow, $\dot{Q}$), as well as a lower percentage of capillaries supporting continuous f_{RBC} , thereby reducing the surface area available for O_2 exchange to the myocyte (Kindig et al., 1999). PH patients often present with lower $\dot{Q}_{TOT}$, however, Tolle and

colleagues (2008) suggest that an impaired systemic oxygen extraction (arteriovenous O_2 difference, $a-vO_2$) during maximal exercise, hypothesizing that, unlike HFrEF, the lower $\dot{V}O_{2\max}$ is not primarily due to decreased $\dot{Q}$, but rather reduced contact between capillaries and contracting myocytes. Based on modeling studies by Federspiel and Popel (1986), this would, in turn, compromise the diffusive component of blood-myocyte O_2 transfer. Furthermore, capillary rarefaction has been implicated in the quadriceps muscle in PH patients (Potus et al., 2014), supporting the potential for decreased surface area available for blood-myocyte O_2 flux, which would lower intramyocyte PO_2 and impair skeletal muscle O_2 utilization (Hogan et al., 1992, Wilson et al., 1977).

The presence of capillary rarefaction, endothelial dysfunction, and reduced $\dot{Q}_{TOT}$ suggests that PH patients may display changes in capillary hemodynamics at rest. Therefore, this study aimed to determine if PH elicits changes in capillary hemodynamics as a putative mechanism for impaired perfusive and diffusive O_2 transport in skeletal muscle in PH. This rationale is based, in part, upon our previous observations in HFrEF (Kindig et al., 1999; rev. Poole et al., 2012). We hypothesized that rats with monocrotaline (MCT)-induced PH would display: 1) reductions in f_{RBC} and RBC velocity (V_{RBC}) in capillaries of resting skeletal muscle, and 2) a lower proportion of capillaries supporting continuous f_{RBC} and thus O_2 delivery to the myocyte. The results of this investigation provide fundamental insights into the currently underexplored field of skeletal muscle microcirculatory function in PH which may support a mechanistic basis for impaired skeletal muscle oxygenation and thus exercise intolerance in patient populations.

Methods

Animals

Experiments were performed on 18 adult (~3-4 months) male Sprague-Dawley rats (~300 g; Charles River Chicago, Illinois, USA). Animals were randomized into the monocrotaline (MCT)-induced PH or healthy control (HC) groups (MCT, n=9; HC, n=9). Upon arrival, animals were maintained in accredited (Association for the Assessment and Accreditation of Laboratory and Animal Care; AAALAC) animal facilities under a 12:12 h light:dark cycle with food and water provided ad libitum. All procedures and protocols were approved by the Kansas State University Institutional Animal Care and Use Committee and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The present investigation contains a partial subset of a separate study (Schulze et al., 2021), but all measurements reported herein are original findings.

Monocrotaline-induced Pulmonary Hypertension

Monocrotaline alkaloid (MCT) was administered via a single intraperitoneal injection at a dosage of 50 mg/kg, which has been shown to induce progressive PH (Gomez-Arroyo et al., 2011; Brown et al., 2015). MCT (Sigma-Aldrich, St. Louis, MO) was dissolved in a solution containing 50% saline and 50% 200-proof ethanol at room temperature and a total of 1 mL of fluid was injected into each rat in the experimental group while HC rats received 1 mL of vehicle (0.5 mL saline and 0.5 mL 200-proof ethanol). Injections were made immediately following pre-injection echoes under anesthesia (see Echocardiography) with a 27 G needle in the lower right quadrant of the abdomen with care taken to avoid internal organs. Importantly, characteristics of PH (i.e., elevations in pulmonary artery pressures, remodeling of small pulmonary arteries, and right ventricular (RV) hypertrophy) occur 3-4 weeks following injection. Animals were monitored weekly via echocardiography along with noting body weight and behavioral changes.

Echocardiography

Transthoracic echocardiography was performed using a commercially available system (Logiq S8; GE Health Care, Milwaukee, WI) with a 13 MHz linear transducer (L4-12t) prior to MCT or saline injection, and then weekly following injection to monitor disease progression. All comparisons were made between the pre-injection measurement and final measurement taken just prior to experimentation. Animals were initially anaesthetized by inhalation of a 5% isoflurane-O₂ mixture and maintained on $\leq 2.0\%$ isoflurane-O₂ (Butler Health Supply, Dublin, OH). Core temperature was maintained at $\sim 37^{\circ}\text{C}$, measured via rectal thermometer. 2-D and M-mode images of the left ventricle (LV) were obtained from the parasternal short axis window and analyzed for end-systolic/diastolic volumes, stroke volume, and ejection fraction as previously described (Craig et al., 2019a). RV measurements were taken at the level of the aortic valve, just proximal to the pulmonary valve, in the short axis view. Pulsed Doppler ultrasound was used to assess pulmonary artery ejection time (ET), acceleration time (AT), and peak velocity, and the ratio of AT to ET (AT/ET) was calculated and used as a clinical parameter for the confirmation of PH (AT/ET < 0.3) (Jones et al., 2002). Upon verification of PH, the following terminal experiments were performed.

Surgical Preparations

All surgeries were performed utilizing the same anesthetic protocol as described above. The right jugular vein and right common carotid artery were surgically isolated and cannulated with a 2-French catheter-tip pressure micromanometer (Millar Instruments, Houston, TX, USA). The micromanometer was advanced into the RV and LV, respectively, to obtain pressures within each ventricle via a PowerLab/LabChart data acquisition system (AD Instruments). Following RV and LV measurements, the micromanometer was withdrawn and the right common carotid artery was catheterized (PE-10 connected to PE-50, Intra-Medic polyethylene tubing; Clay

Adams Brand; Becton Dickinson, Sparks, MD) for measurement of mean arterial pressure (MAP) and heart rate (HR). The caudal artery was surgically isolated and catheterized for administration of pentobarbital sodium. Rats were then progressively transitioned from isoflurane onto pentobarbital sodium (50 mg/kg). Depth of anesthesia was continuously monitored via toe pinch and palpebral reflexes with pentobarbital sodium supplemented as deemed necessary throughout the remaining experimental protocols.

Intravital Microscopy

Incisions were made to expose the left spinotrapezius muscle, with overlying skin and fascia reflected as previously described (Hirai et al., 2018). The spinotrapezius was chosen for its muscle fiber-type composition and citrate synthase activity, being closely analogous to the untrained human quadriceps muscle (Delp & Duan, 1996; Leek et al., 2001). The spinotrapezius was superfused with warmed ($\sim 37^{\circ}\text{C}$) Krebs-Henseleit bicarbonate-buffered solution equilibrated with 5% CO_2 -95% N_2 at pH 7.4, and exposed tissue was protected with Saran Wrap (Dow Brands, Indianapolis, IN) to minimize dehydration. The muscle was carefully dissected and sutured (6-0 silk) around the caudal periphery to a thin wire horseshoe-shaped manifold at five to six equidistant points. The rat was then placed on a water circulation-heated (38°C) Lucite platform with the spinotrapezius positioned such that a microvascular field, midway between arteriolar and venular ends within the dorsal surface, could be observed using an intravital microscope (Nikon, Eclipse E600-FN; $\times 40$ objective; 0.8 numerical aperture) equipped with a noncontact, illuminated lens, and a high resolution color monitor (total viewing area = $270 \times 210 \mu\text{m}$; Sony Trinitron PVM-1954Q, Ichinonya, Japan). The muscle was transilluminated to ensure clear resolution of the sarcomere A-bands within one-third to two-thirds of the muscle fibers. The final magnification ($\times 1,184$) was confirmed by initial calibration of the system using a stage

micrometer (MA285, Meiji Techno). This magnification is adequate for measuring all essential structural and hemodynamic variables (Poole et al., 1997; Kindig et al., 1999). The manifold was attached to a muscle stretching apparatus that permitted precise length changes along the longitudinal axis of the muscle. Sarcomere length was approximated to $<2.7\ \mu\text{m}$ for physiological relevance throughout the observation period, and the muscle continuously superfused with the Krebs-Henseleit solution. Once the spinotrapezius muscle was positioned on the platform, 8–10 microvascular viewing fields were each recorded for ~60-180 seconds for every animal. Recordings were time-referenced and fields and stored for subsequent offline analysis. MAP was monitored throughout the duration of data acquisition. Arterial blood samples (0.5 mL) were taken at the end of microcirculatory measurements for arterial pH, PaCO_2 , PaO_2 , systemic hematocrit, and lactate concentration.

Capillary Data Analysis

Five to seven fields demonstrating clear visualization of sarcomeres, fibers, and capillaries were chosen for analysis from each rat at random. Examination of the microvascular fields was conducted in real time and by frame-by-frame analysis techniques (Dartfish Video Software, Fribourg, Switzerland). Muscle fiber width was measured along a line perpendicular to the muscle fiber axis for each muscle fiber. Sarcomere length was calculated from sets of 10 consecutive sarcomeres (i.e., distance between 11 consecutive A-bands) measured parallel to the muscle fiber longitudinal axis. This measurement was repeated 3–4 times where sarcomeres were visible to obtain a mean sarcomere length for each viewing field. Capillary lineal density was determined as the number of capillaries crossing a line drawn perpendicular to the fiber axis and expressed per mm. Capillaries supporting RBC flow were assessed in real time, and each capillary was placed into one of three categories: 1) normal flow = 30 s of continuous, 2)

intermittent flow = impeded flow for <10 of 30s, or 3) stopped flow = impeded flow or stopped flow for >10 of 30 s (i.e., stationary RBCs). These criteria determined the percentage of continuously flowing capillaries in a field of view [i.e., (number of capillaries supporting RBC flow/total number of visible capillaries per area) × 100]. Capillary luminal diameter (d_c) was measured in all capillaries in which hemodynamics were assessed and where the capillary endothelium was clearly visible on both sides of the lumen (2–4 measurements/capillary) with calipers accurate to ± 0.25 mm (± 0.17 μ m at $\times 1,184$ magnification). V_{RBC} was calculated in all capillaries that were continuously RBC perfused and in which the RBC could be followed over several frames and RBC path length measured. f_{RBC} was determined by counting the number of RBCs in a capillary which passed an arbitrary point. For each capillary in which hemodynamic data were measured, capillary tube hematocrit (Hct_{cap}) was calculated by the following equation:

$$Hct_{cap} = (RBC \text{ volume} \times f_{RBC}) / [\pi \times (d_c/2)^2 \times V_{RBC}]$$

where RBC volume was taken to be $61 \mu\text{m}^3$ (Altman & Dittmer, 1974) and capillaries were approximated as circular in cross section (Kindig et al., 1998).

Postmortem Measurements

While under a deep plane of anesthesia, all rats were euthanized via pentobarbital sodium overdose (>100 mg/kg, i.a) followed by a bilateral pneumothorax. The RV, LV, lungs, diaphragm, and spinotrapezius muscle, were dissected and weighed. The right lower leg of each rat was exposed, and the tibial bone was isolated, removed, and the length of the bone measured. RV hypertrophy was determined via the Fulton index: RV weight (mg) / LV + septum (S) weight (mg) (Fulton et al., 1952). RV hypertrophy was also expressed as RV/body weight and RV/tibia bone length.

Data and Statistical Analyses

Student paired (within animal) and independent two-sample (between HC and MCT) t-tests were utilized to determine differences in morphometric, Millar, and hemodynamic measurements, followed by Pearson correlation tests and linear regression analyses of these data. Mann Whitney tests were used when data were nonparametric. Echocardiographic data was analyzed via two-way repeated measures Analysis of Variance (ANOVA). Data are presented as mean $\pm$ standard deviation. Significance was set at $P < 0.05$.

Results

Morphometric and Echocardiographic Data

Eighteen rats were analyzed for morphometric and echocardiographic data (HC, $n = 9$; MCT, $n = 9$). No differences were observed in pre-injection body weight (HC: 307 ± 31 vs. MCT: 302 ± 26 g; $P > 0.05$) or echocardiographic measurements ($P > 0.05$; Table 2.1). However, final body weight was lower in MCT rats (HC: 445 ± 35 vs. MCT: 379 ± 24 g; $P < 0.05$), therefore tibial bone length was used for relative comparisons of lung mass between groups. Left ventricular echocardiographic measurements did not differ between groups ($P > 0.05$ for all; Table 2.1). MCT rats displayed hallmarks of PH including elevated right ventricular systolic pressure (Table 2.1), increased lung weight normalized to tibial bone length (Table 2.2), RV hypertrophy (Table 2.2), and a reduced acceleration time to ejection time ratio in the pulmonary artery (Table 2.1) compared to healthy rats (all $P < 0.05$). In addition, the MCT rats demonstrated a significant reduction in LV fractional shortening in their final echocardiogram when compared to pre-injection measurements and a lower LV dp/dt compared to healthy (Table 2.1). However, these rats did not exhibit any signs of overt LV failure (i.e., significant reductions in LV ejection fraction or increases in LVEDP; Table 2.1). These characteristics support the development of

PH, pulmonary congestion, and RV compensation preceding overt RV or LV heart failure in the MCT group.

Intravital Microscopy

Seventeen rats (HC, n = 9; MCT, n = 8) were analyzed for capillary hemodynamics. One rat from the MCT group was excluded due to insufficient clarity to measure the desired variables (i.e., f_{RBC} , V_{RBC} , and Hct_{cap}). The proportion of capillaries flowing and sarcomere length were still measured in this animal and included in the final data set. Capillary lineal density was measured only when there was sufficient clarity to count total number of capillaries in a given field. Therefore, lineal density was calculated in a subset of fifteen rats (HC, n = 7; MCT, n = 8).

Spinotrapezius sarcomere length was not different between groups ($2.5 \pm 0.2 \mu m$ for both MCT and HC). The mean capillary diameter, lineal density, and muscle fiber width were not different in MCT compared to HC (Table 2.3). Heart rate was elevated in MCT rats (HC: 361 ± 23 vs MCT: 403 ± 25 bpm; $P = 0.010$), but perfusion pressures (i.e., MAP) did not differ between groups throughout the duration of data acquisition (HC: 104 ± 15 vs. MCT: 102 ± 16 mmHg). No differences were present between groups for arterial blood pH (HC: 7.43 ± 0.04 vs. MCT: 7.38 ± 0.07 ; $P = 0.09$), $PaCO_2$ (HC: 32 ± 3 vs. MCT: 29 ± 4 mmHg; $P = 0.13$), systemic hematocrit (HC: 33 ± 3 vs. MCT: $33 \pm 5\%$; $P = 0.86$), or lactate (HC: 1.01 ± 0.33 vs. MCT: 1.02 ± 0.29 mmol/L; $P = 0.94$); however PaO_2 (HC: 81.9 ± 9.4 vs. MCT: 68.7 ± 10.0 mmHg; $P = 0.01$) and SaO_2 (HC: 94.5 ± 3.0 vs. MCT: $89.0 \pm 8.5\%$; $P = 0.045$) were lower in MCT rats. MCT rats demonstrated a 30% reduction in the proportion of capillaries that supported continuous f_{RBC} and a higher percentage of capillaries that contained temporarily (>10 s) stopped RBCs or that did not support any f_{RBC} over the entire window of observation (Figure 2.1). In the capillaries supporting continuous RBC flow, MCT rats displayed a substantially lower V_{RBC} and

f_{RBC} (Figure 2.2) compared to HC (both $P < 0.05$) but Hct_{cap} did not differ between groups (Table 2.3). There was a significant linear correlation between f_{RBC} and V_{RBC} in all animals ($P < 0.05$; Figure 2.3). The product of the percentage of continuously flowing capillaries and f_{RBC} measures continuous O_2 delivery ($\dot{Q}\text{O}_2$) within a given field sampled. There was an average ~50% decrease in $\dot{Q}\text{O}_2$ to resting skeletal muscle in MCT versus healthy animals ($P < 0.05$; Figure 2.4). Notably, f_{RBC} was negatively linearly correlated with structural ($\text{RV}/(\text{LV}+\text{S})$) and functional (RVSP) indicators of PH severity (both $P < 0.05$; Figures 2.5A-B).

Discussion

This investigation assessed, for the first time to our knowledge, skeletal muscle capillary hemodynamics in MCT-induced PH compared to healthy rats. The distribution of skeletal muscle blood flow within the capillary network has not previously been examined and is crucial to understanding O_2 exchange in PH. The primary original findings of this study support the notion that resting skeletal muscle blood flow is reduced in MCT-induced PH, occurring via a reduction in both V_{RBC} and f_{RBC} . Further, the proportion of non-flowing capillaries (i.e., zero f_{RBC}) is markedly increased in MCT animals. These findings support the hypotheses that both perfusive and diffusive mechanisms of microvascular O_2 transport are compromised in PH, offering valuable insights for skeletal muscle dysfunction in PH.

Validation of MCT-induced PH

Confirmation of moderate PH was assessed using clinically established measurements (AT/ET, RVSP; Jones et al., 2002; Kitabatake et al., 1983) as well as morphometric data consistent with previous investigations in the MCT rat model used herein (Brown et al., 2015; 2017; Schulze et al., 2021). Our laboratory recently demonstrated that the dose of MCT (50 mg/kg) used herein elicits reproducible PH development within ~3 weeks in male Sprague-

Dawley rats (Schulze et al., 2021). Tables 2.1 and 2.2 demonstrate that the animals in the present investigation displayed hallmarks of PH progression (i.e., $\uparrow$ RV/(LV+S), $\uparrow$ lung weight, $\downarrow$ AT/ET, $\uparrow$ RVSP) within 25 ± 9 days, consistent with previous literature (Gomez-Arroyo et al., 2011). Importantly, this model induces moderate PH, indicating that the alterations in the skeletal muscle microcirculation presented herein occur prior to and independent of distinct RV or LV failure as indicated by lack of changes in LV ejection fraction and LVEDP.

Hemodynamics

Surgical exteriorization of the spinotrapezius muscle in vivo was performed as previously described which limits disruption of neural and vascular supply and induces minimal fascial disturbance to prevent muscle damage (Gray, 1973; Bailey et al., 2000; Poole et al., 1997; Kindig et al., 1999). This preparation has been performed in healthy rats in addition to heart failure, aging, and diabetes and facilitates collection of high resolution capillary structural and hemodynamic variables which are consistent across healthy control animals in previous studies and within the present investigation (Kindig et al., 1999; Padilla et al., 2006; Copp et al., 2009; Hirai et al., 2021). Sarcomere length was maintained at $<2.7 \mu\text{m}$ to prevent stretch-induced functional or structural alterations, as previously studied (Poole et al., 1997). In addition, healthy animals in this investigation presented with a majority of capillaries ($>80\%$) flowing (Figure 2.1), as is expected in healthy resting skeletal muscle (Poole et al., 2011; Poole, 2019; Mendelson et al., 2021).

Consistent with our hypotheses, MCT rats demonstrated substantial decrements in capillary hemodynamics (i.e., reduced V_{RBC} , f_{RBC} , and percentage of capillaries supporting continuous flow) at rest compared to healthy controls, suggesting arteriolar and/or microcirculatory impairments in skeletal muscle. V_{RBC} and f_{RBC} represent the perfusive

component of $\dot{O}_2$ and substrate exchange, with such decreases indicating reduced muscle $\dot{Q}O_2$. It is possible that lower $\dot{Q}_{TOT}$ could explain, in part, the decreased V_{RBC} and f_{RBC} ; however, it is important to note that stroke volume was only marginally less in MCT animals compared to HC ($P=0.131$) and heart rate tended to be elevated. Consistent with the findings of Tolle et al. (2008), this suggests that the changes in $\dot{Q}_{TOT}$ alone would not be sufficient to lower muscle V_{RBC} and f_{RBC} in PH. This suggests the presence of arteriolar vasoconstriction to the quiescent spinotrapezius. The present investigation could not determine whether reductions are present in V_{RBC} and f_{RBC} during contractions. However, our previous observations in HFrEF rats demonstrated that capillaries not supporting f_{RBC} at rest failed to resume flowing during contractions (Richardson et al., 2003). Importantly, the proportional decrements in V_{RBC} and f_{RBC} (Figure 2.2) maintained capillary hematocrit at ~19% irrespective of PH such that, although perfusive conductance was decreased, the O_2 diffusing capacity per flowing capillary would not be expected to change with PH.

As alluded to above, one potential explanation for the lower V_{RBC} and f_{RBC} , which were reduced in proportion to the cardiac structural (Figure 2.5A) and functional (Figure 2.5B) derangements of PH, lies within the arteriolar network, which controls blood flow distribution within the capillary beds. Arteriolar control may be disrupted due to dysregulation of vasoactive substances including prostacyclin, endothelin-1, and nitric oxide (Humbert et al., 2004), or via systemic arterial stiffening (Radchenko et al., 2020), all of which have been implicated in the pathophysiology of PH. Such disturbances in skeletal muscle arteriolar function could impair vasodilation and/or facilitate chronic increases in vascular tone. Peripheral endothelial dysfunction has also been suggested in PH and, as the endothelium is responsible for sensing changes in shear stress and responding to vasoactive mediators, this could represent an additional

limitation to perfusive O_2 conductance (Peled et al., 2008; Wolff et al., 2007). Furthermore, increased sympathetic nerve activity with PH could exacerbate redistribution of blood flow away from skeletal muscle (Velez-Roa et al., 2004). Any of these mechanisms, independently or in concert, would effectively lower perfusion to this microvascular bed at rest, resulting in such reductions in V_{RBC} and f_{RBC} .

Importantly, the percentage of capillaries flowing in healthy skeletal muscle at rest has been shown to be >80% (Poole et al., 2011; Poole, 2019; Mendelson et al., 2021). Our healthy values herein corroborate these previous findings; however, there was a 30% reduction in the proportion of continuously perfused capillaries in the MCT group. Three potential mechanisms exist to explain the increased proportion of stopped capillaries in MCT rats: 1) As mentioned above, compromised arteriolar function due to impaired smooth muscle or endothelium, or alterations in levels of vasoactive substances limiting the capacity for proper arteriolar control, 2) PH increases pulmonary artery and RV pressures and consequently induces venous congestion (increased central venous pressure, CVP). Because the pressure differential (ΔP) across skeletal muscle capillaries is extremely small (Mendelson et al., 2021), elevated CVP would decrease capillary flow, 3) As a compensatory mechanism to constrain the fall in f_{RBC} and preserve Hct_{cap} . Should all capillaries remain flowing, this would necessitate a greater magnitude of the fall in V_{RBC} , f_{RBC} , and perhaps Hct_{cap} in order to perfuse all flowing capillaries.

The 40% decrease in V_{RBC} (in concert with reduced f_{RBC} ; Figure 2.2) lowers perfusive O_2 conductance, but it will also serve to lengthen RBC transit time through the capillary, allowing for greater fractional O_2 extraction. Indeed, it is by this mechanism that fractional O_2 extraction is elevated in left ventricular heart failure (i.e., HFrEF; Kindig et al., 1999). If extraction is represented by the formula (Roca et al., 1992):

$$\% \text{ O}_2 \text{ extraction} = 1 - e^{-\text{DmO}_2 / \beta \dot{Q}_m}$$

where DmO_2 is muscle O_2 diffusing capacity, β is the slope of the O_2 dissociation curve in the physiologically relevant range, and $\dot{Q}_m$ is muscle blood flow, a reduction in $\dot{Q}_m$ would be expected to increase fractional extraction in the absence of a concomitant lowering of DmO_2 . As DmO_2 is determined by the surface area contact between the RBCs in the flowing capillaries and adjacent myocytes, this would be dictated in an individual capillary by Hct_{cap} . Akin to HFrEF (Kindig et al., 1999), Hct_{cap} was not different between healthy and MCT animals, but the lowered perfusive O_2 conductance would necessitate an increased fractional O_2 extraction in capillaries supporting f_{RBC} . Despite this, the lower proportion of flowing capillaries in MCT rats would reduce the total surface area contact between the RBC and the myocyte within the muscle at any given instant, limiting overall DmO_2 (Wagner et al., 1997). Such heterogeneity among capillaries (i.e., some displaying increased extraction while others support no O_2 flux) may, in part, explain the unchanged level of whole muscle O_2 extraction, reflected by resting interstitial oxygen pressures in the spinotrapezius ($\text{PO}_{2\text{is}}$) (Wagner et al., 1997; Schulze et al., 2021).

Notwithstanding this occurrence, to sustain a given $\dot{V}\text{O}_2$, fractional O_2 extraction must be substantially increased in the individual capillaries supporting flow. According to the Fick's principle, estimations for resting muscle $\dot{V}\text{O}_2$ herein are ~36% lower in MCT rats, suggesting that such elevations in fractional O_2 extraction are insufficient to compensate for the lower f_{RBC} and increased proportion of capillaries which are not participating in O_2 exchange.

Experimental Considerations

1) Females display a higher incidence of PH and, interestingly, better survivability (Lahm et al., 2014) and the cardioprotective nature of estrogen may provide some insights into this phenomenon. As the present investigation focuses on male rats, future studies examining both

female and ovariectomized female rats should assess sexual dimorphism in microcirculatory hemodynamics, and whether these potential differences may be mediated by estrogen. 2) Transillumination microscopy was chosen for its ability to visualize capillaries within the skeletal muscle in real time while minimizing tissue damage. It is possible that, using this technique, we were unable to visualize all capillaries within a given field of view. However, as image clarity and lineal density were not obviously different between groups, it is unlikely that the inability to visualize individual capillaries played any substantial role in the capillary hemodynamics results herein. 3) Although we examined only a relatively modest area of tissue per screen (270 x 210 μm), several screens and multiple capillaries per screen were analyzed, wherein we detected no significant differences across screens within a given muscle in any of the primary measurements. Blood flow data using fluorescent microspheres in skeletal muscle have not yet been reported in the MCT model and would be a valuable future direction for linking the microvascular results in the present investigation to bulk tissue blood flow. 4) The MCT rat model allows mechanistic study of PH and elicits reliable hallmarks of disease such as pulmonary vascular remodeling and subsequent RV hypertrophy (Gomez-Arroyo et al., 2011). The data herein may provide insights into the underlying bases for skeletal muscle impairments documented in human patients. While off-target effects of MCT on skeletal muscle have not been determined, the metabolism of MCT allows it to primarily affect the pulmonary vasculature, mirroring the human pathology and, to our knowledge, MCT does not affect peripheral mitochondrial or vascular function directly. Nonetheless, translation of animal research to humans should be employed with caution. Additionally, it is important to note that the present investigation focused on moderate PH. With increased duration of pathology

following MCT injection and thus greater disease severity, structural microvascular changes not seen herein may potentially develop.

Conclusions

MCT-induced PH in male rats lowers resting microvascular skeletal muscle blood flow via reduced perfusive (i.e., V_{RBC} and f_{RBC}) and diffusive (proportion of flowing capillaries) O_2 transport, possibly due to upstream arteriolar dysregulation. Despite this, the lengthening of RBC capillary transit time, concurrent with unchanged Hct_{cap} , are expected to elevate muscle fractional O_2 extraction in the individual capillaries supporting flow which would allow the muscle to sustain a given metabolic rate. These data support the notion that capillary hemodynamics in quiescent skeletal muscle are impaired in MCT-induced PH and provide novel insights into potential mechanisms of skeletal muscle dysfunction in PH.

Funding: This work was supported in part by the Sustained Momentum for Investigators with Laboratories Established (SMILE) Grant awarded to T.I.M by Kansas State University College of Veterinary Medicine. T.D.C was financially supported by the Ruth L. Kirschstein National Research Service Award F31HL145981.

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Table 2.1 Echocardiographic and Millar catheter measurements.

	Healthy		MCT	
	Pre	Final	Pre	Final
<u>Echocardiographic</u>				
PA ET (ms)	85 ± 8	82 ± 6	79 ± 5	87 ± 6
PA AT (ms)	30 ± 2	29 ± 4	28 ± 2	19 ± 3 *, #
PA Peak Velocity (cm/s)	90 ± 19	101 ± 15	97 ± 16	95 ± 10
PA AT/ET	0.37 ± 0.07	0.35 ± 0.04	0.36 ± 0.04	0.22 ± 0.05 *, #
LV Stroke Volume (mL)	0.54 ± 0.14	0.76 ± 0.18 #	0.58 ± 0.16	0.63 ± 0.18
LV Ejection Fraction (%)	84 ± 5	85 ± 7	87 ± 5	81 ± 7
LV Fractional Shortening (%)	49 ± 5	54 ± 13	58 ± 14	45 ± 7 #
<u>Millar Catheter</u>				
RVSP (mmHg)	-	21 ± 8	-	53 ± 15 *
LVEDP (mmHg)	-	11 ± 4	-	8 ± 3
LV dP/dt (mmHg/s)	-	7184 ± 1467	-	5855 ± 870 *

Data are means ± SD. Healthy (n = 9), monocrotaline (MCT, n = 9). PA, pulmonary artery; ET, ejection time; AT, acceleration time; LV, left ventricle; RVSP, right ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; LV dP/dt, rise in pressure over time. Reduced PA AT and AT/ET determined disease progression (criteria for terminal studies). This was accompanied by increases in RVSP. * P < 0.05 vs. healthy, # P < 0.05 vs. pre-injection within group.

Table 2.2 Morphometric data.

	Healthy	MCT	P-value
RV (mg)	249 ± 29	365 ± 88 *	0.005
LV (mg)	888 ± 122	830 ± 117	0.316
Lungs (mg)	1403 ± 220	2321 ± 277 *	< 0.001
Diaphragm (mg)	967 ± 134	876 ± 139	0.174
RV/(LV+S) (mg/mg)	0.28 ± 0.05	0.44 ± 0.11 *	< 0.001
RV/BW (mg/g)	0.56 ± 0.06	0.96 ± 0.23 *	0.001
RV/Tibia (mg/mm)	6.0 ± 0.6	8.5 ± 2.0 *	0.010
Lung/BW (mg/g)	3.14 ± 0.32	6.15 ± 0.83 *	< 0.001
Lung/Tibia (mg/mm)	32.7 ± 4.4	53.0 ± 8.0 *	< 0.001
Diaphragm/BW (mg/g)	2.17 ± 0.24	2.30 ± 0.28	0.306
Diaphragm/Tibia (mg/mm)	22.7 ± 2.8	20.3 ± 4.1	0.204

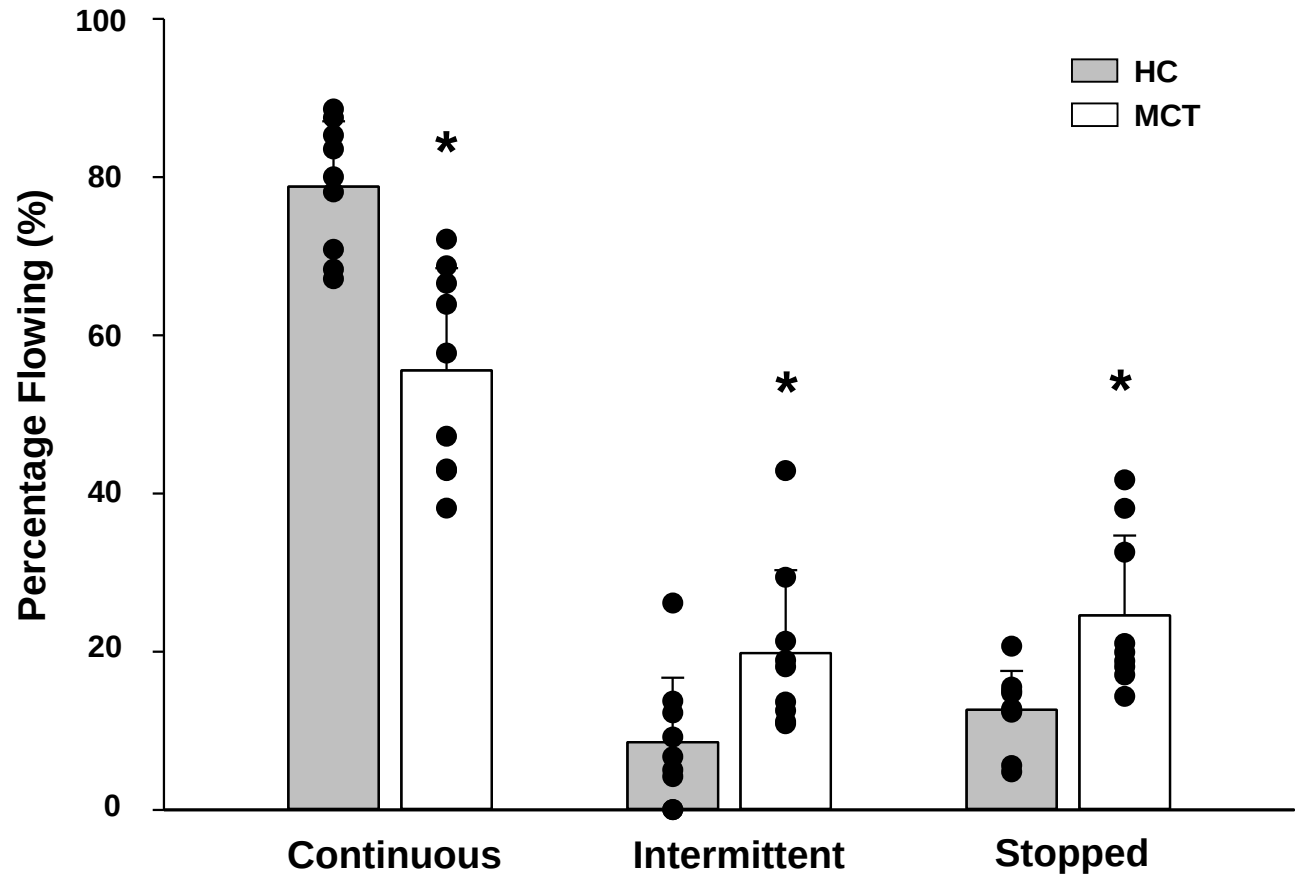
Data are means ± SD. Healthy (n = 9), monocrotaline (MCT, n = 9). RV, right ventricle; LV + S, left ventricle plus septum; BW, body weight; Tibia, tibial bone length. * P < 0.05 vs. healthy.

Table 2.3 Capillary measurements.

	Healthy	MCT	P-value
Hct _{cap} (%)	19 ± 4	19 ± 4	0.975
Capillary Diameter (µm)	5.8 ± 0.7	5.7 ± 0.5	0.694
Lineal Density (capillaries/mm)	36 ± 5	33 ± 3	0.131
Muscle Fiber Width (µm)	64 ± 5	62 ± 6	0.381

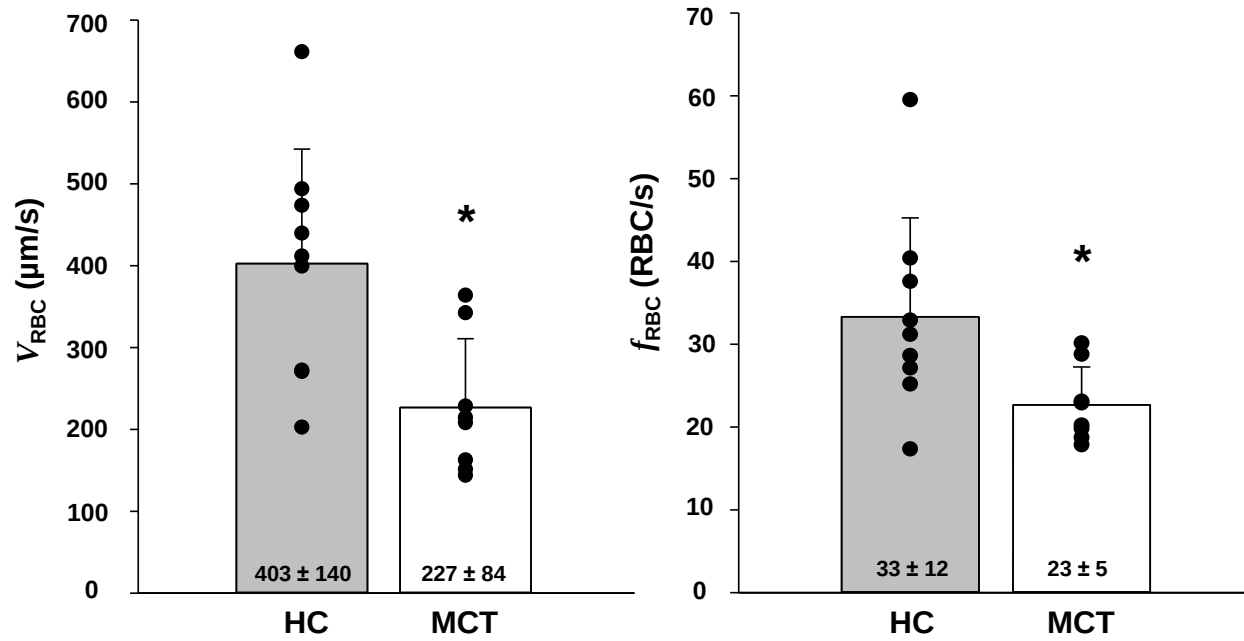
Data are means ± SD. Healthy (n = 9), monocrotaline (MCT, n = 8) for Hct_{cap}, capillary hematocrit.

Figure 2.1 Percentage of flowing capillaries.



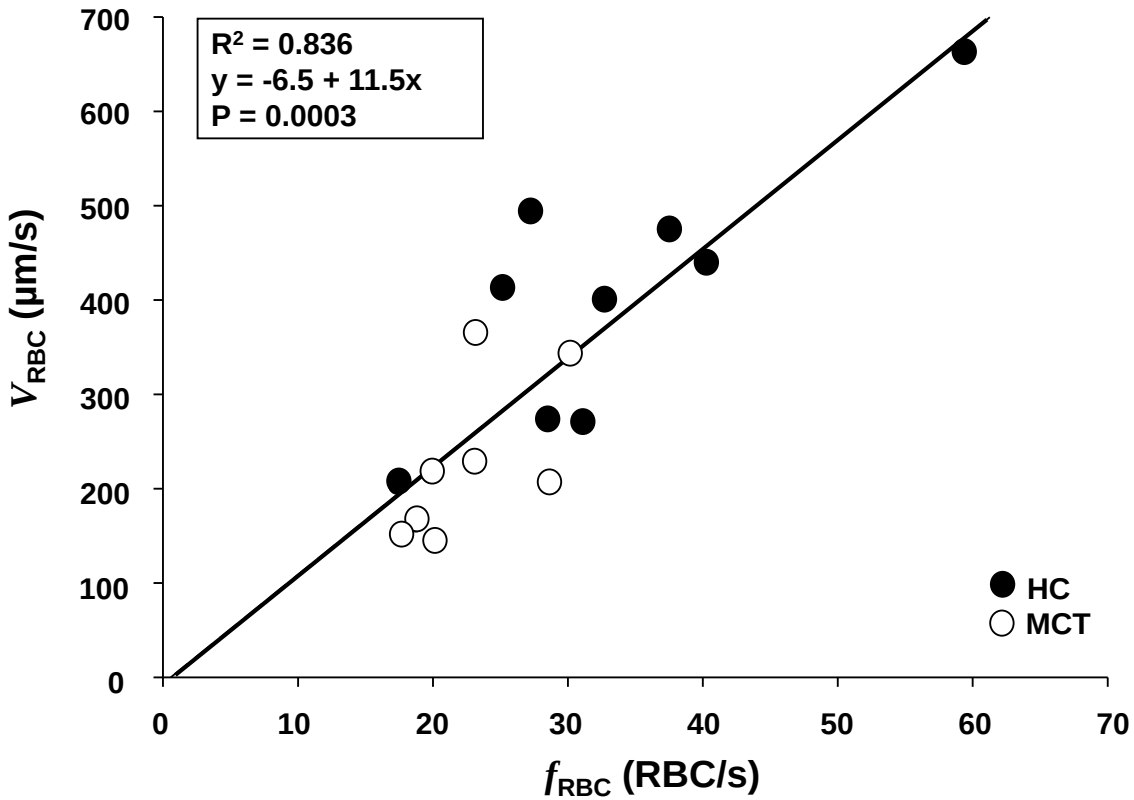
Percentage of capillaries supporting continuous and intermittent red blood cell (RBC) flow and stopped capillaries in healthy (HC, n = 9) and monocrotaline (MCT, n = 9) rats. * P < 0.05 versus HC.

Figure 2.2 Red blood cell flux and velocity.



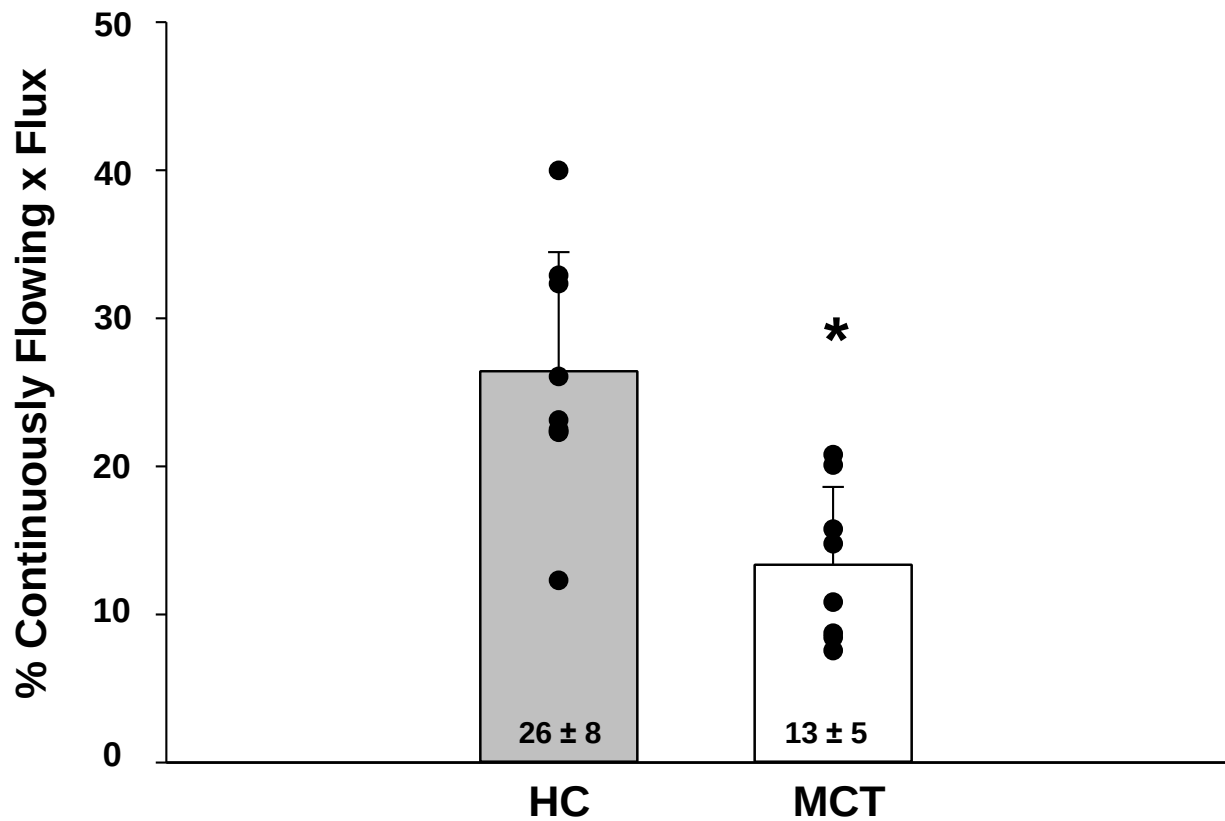
Comparison of red blood cell (RBC) hemodynamics (velocity, V_{RBC} and flux, f_{RBC}) in muscles of healthy (HC, $n = 9$) and monocrotaline (MCT, $n = 8$) rats. * $P < 0.05$.

Figure 2.3 Correlation between red blood cell flux and velocity.



Relationship between RBC velocity (V_{RBC}) and flux (f_{RBC}) in healthy controls (HC, $n = 9$) and monocrotaline (MCT, $n = 8$). Note that V_{RBC} and f_{RBC} increase or decrease in proportion to one another to maintain a given hematocrit. The slope of the solid line dictates hematocrit.

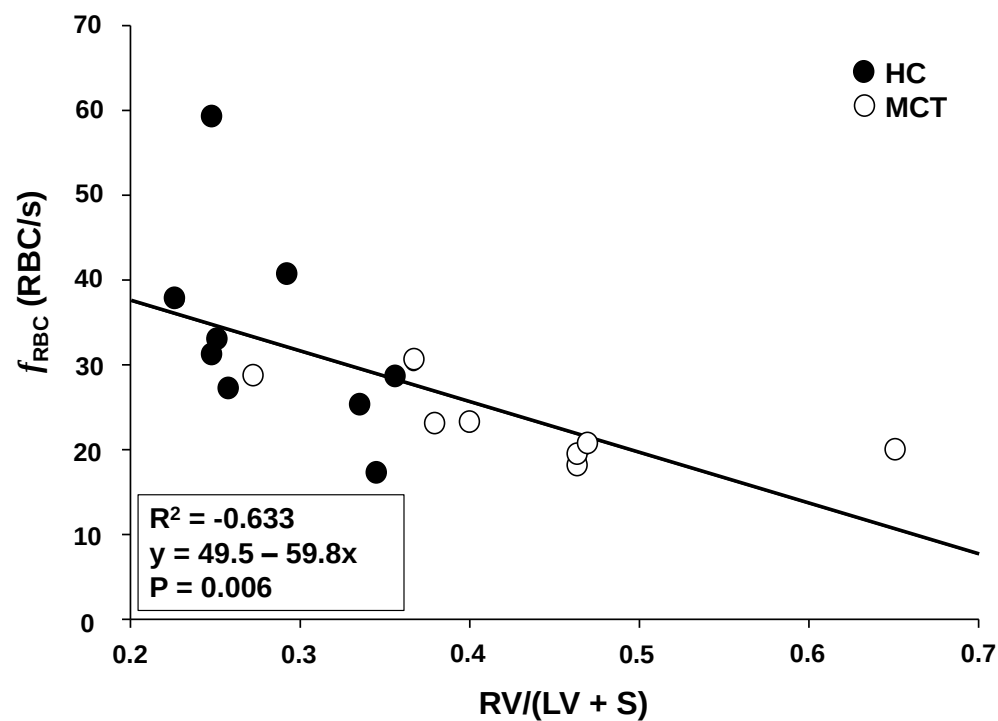
Figure 2.4 Muscle O₂ delivery index.



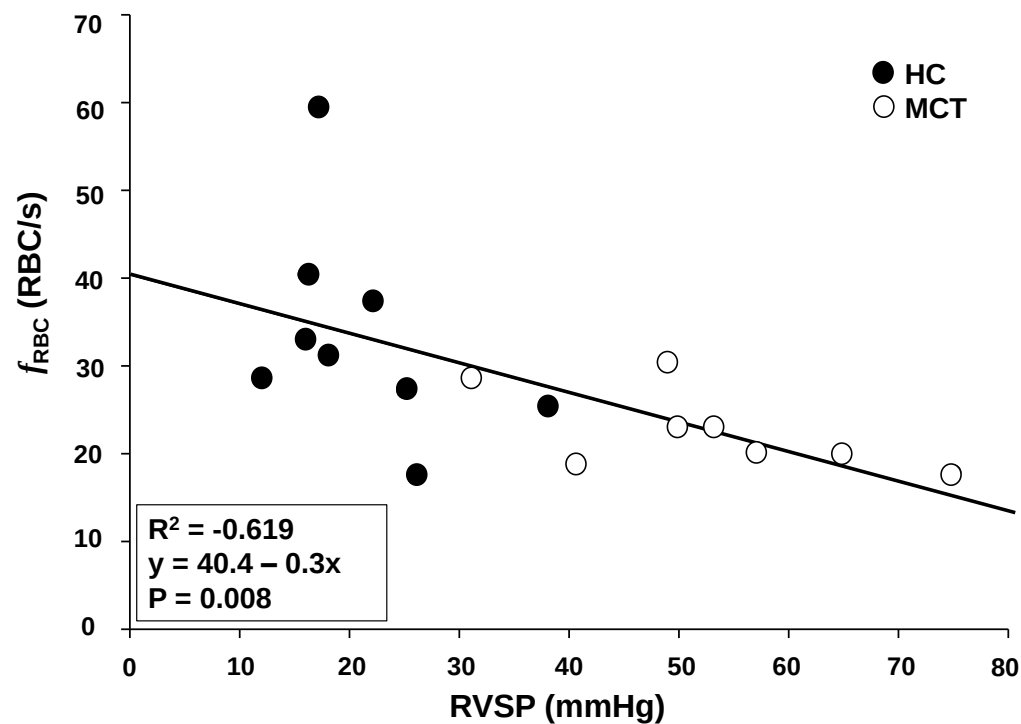
Index of continuous O₂ delivery to muscle in healthy (HC, n = 9) and monocrotaline (MCT, n = 8) animals. * P < 0.05.

Figure 2.5 Relationship between capillary hemodynamics and disease severity.

A



B



Relationship between red blood cell (RBC) flux (f_{RBC}) and structural (**A**) and functional (**B**) measurements of PH severity. RV/(LV+S), right ventricular weight/weight of left ventricle + septum; RVSP, right ventricular systolic pressure.

Chapter 3 - Pulmonary Hypertension Alters Blood Flow

Distribution and Impairs the Hyperemic Response in the Rat

Diaphragm

Abstract

Pulmonary hypertension (PH) is characterized by pulmonary vascular remodeling, respiratory muscle and cardiac impairments, and exercise intolerance. Specifically, impaired gas exchange increases work of the diaphragm; however, compromised contractile function precludes the diaphragm from meeting the increased metabolic demand of chronic hyperventilation in PH. Given that muscle contractile function is in part, dependent upon adequate blood flow ($\dot{Q}$), diaphragmatic dysfunction may be predicated by an inability to match oxygen delivery with oxygen demand. We hypothesized that PH rats would demonstrate a decreased hyperemic response to contractions compared to healthy controls. Methods: Sprague-Dawley rats were randomized into healthy (HC, n=7) or PH (n=7) groups. PH rats were administered monocrotaline (MCT) while HC rats received vehicle. Disease progression was monitored via echocardiography. Regional and total diaphragm blood flow and vascular conductance at baseline and during 3 minutes of electrically-stimulated contractions were determined using fluorescent microspheres. Results: PH rats displayed morphometric and echocardiographic criteria for disease (i.e., acceleration time/ejection time, right ventricular hypertrophy). In all rats, total costal diaphragm $\dot{Q}$ increased during contractions and did not differ between groups. In HC rats, there was a greater increase in medial costal $\dot{Q}$ compared to PH rats (55 ± 3 vs. $44 \pm 4\%$, $P < 0.05$), who demonstrated a redistribution of $\dot{Q}$ to the ventral costal region. Conclusions: These findings support a redistribution of regional diaphragm perfusion and an impaired medial costal hyperemic response in PH, suggesting that PH alters diaphragm vascular function and oxygen delivery, providing a potential mechanism for PH-induced diaphragm contractile dysfunction.

Introduction

Pulmonary hypertension (PH) is a chronic disease characterized by elevated pulmonary arterial pressures and structural remodeling of the pulmonary vasculature resulting in dyspnea, cardiac dysfunction, and premature death. PH can occur idiopathically or as a comorbidity, however, all PH etiologies lower exercise tolerance and quality of life [Halank et al., 2013]. The pathological changes in the pulmonary vasculature with PH can induce pulmonary congestion and poor gas exchange, which culminate in an increased ventilatory demand [Sun et al., 2003; Laveneziana et al., 2013] and consequently, increased work of the primary respiratory muscle, the diaphragm. Impaired pulmonary function and ventilatory insufficiency [Sun et al., 2001; Sun et al., 2003] largely contribute to dyspnea at rest and during exercise, therefore, understanding the consequences of heightened work of the diaphragm in PH are integral to improving exercise capacity and quality of life for PH patients. Previous investigations in PH patients [Meyer et al., 2005; Kabitz et al., 2008; Laveneziana et al., 2013; Breda et al., 2014; Spiesshoefer et al., 2019] and animal models of PH [de Man et al., 2011; Manders et al., 2012; Ahn et al., 2013; Himori et al., 2017] demonstrate diaphragm weakness and contractile dysfunction. Presently, the precise mechanisms responsible for PH-induced diaphragm dysfunction remain unknown.

It was initially believed that PH-induced systemic dysfunction occurred solely due to cardiopulmonary pathology. While central dysfunction undoubtedly plays a substantial role, it is becoming evident that pathological changes extend beyond these central manifestations, specifically in skeletal muscle. Impaired strength, oxygenation, and mitochondrial function in peripheral skeletal muscle [Bauer et al., 2007; Mainguy et al., 2010; Schulze et al., 2021; Schulze et al., 2022; Malenfant et al., 2015a; Malenfant et al., 2015b; Dimopoulos et al., 2013] independent of reduced cardiac output support the notion of vascular dysfunction beyond the

pulmonary circulation. It has been suggested that diaphragm contractile dysfunction precedes central hemodynamic compromise [Kosmas et al., 2022], however, the effects of PH on bulk and regional diaphragm perfusion have not been investigated directly. In order to sustain diaphragmatic contractile function, there must be a tight coupling of O₂ delivery ($\dot{Q}O_2$)-to-O₂ demand ($\dot{V}O_2$). Given that $\dot{V}O_2 \propto \dot{Q}O_2 \times PO_2$, inadequate $\dot{Q}O_2$ to maintain $\dot{V}O_2$ will necessitate a lower intramyocyte PO₂, possibly inducing metabolic perturbations which hasten diaphragm muscle fatigue [Hogan et al., 1992; Poole et al., 1997; Poole et al., 2001; Davis et al., 2012]. Whilst the diaphragm has properties that distinguish it from other skeletal muscle (e.g., continually active, high oxidative and vasodilatory capacities [Poole et al., 2000], countercurrent capillary flow [Kindig & Poole, 1998]), blood flow dysregulation in the hyperactive PH diaphragm [Laveneziana et al., 2013] could nevertheless result in local areas of hypoxia, which would thereby play a pivotal role in the increased oxidative stress [Himori et al., 2017] and decreased force generation [de Man et al., 2011] associated with PH-induced diaphragm dysfunction. Peripheral endothelial dysfunction has been demonstrated in PH [Wolff et al., 2007; Peled et al., 2008] and, if present in the diaphragm, would reduce the hyperemic response, exacerbating exercise intolerance and providing a putative mechanism for diaphragmatic contractile impairments and dyspnea in PH.

Therefore, the overall objective of this study was to investigate the effects of monocrotaline (MCT)-induced PH on bulk and regional diaphragmatic blood flow and vascular conductance. We tested the hypotheses that in MCT rats with moderate severity PH versus healthy control there will be: 1) lower diaphragm vascular conductance and blood flow, and 2) a decreased diaphragm hyperemic response to 1 Hz contractions. Additionally, this question will provide insights to the mechanistic bases for the diaphragm dysfunction associated with PH.

Methods

Animals

Fourteen female Sprague-Dawley rats (4-6 months old) were obtained from Charles River Laboratories (Boston, MA, USA) for this investigation. The greater prevalence of PH in females [Badesch et al., 2010; Hester et al., 2019], in combination with the paucity of literature examining females with PH, provides support for our use of female rats herein. Rats were randomized into healthy control (HC, n=7) and monocrotaline (MCT)-induced pulmonary hypertension animals (MCT, n=7). The Sprague-Dawley rat was chosen due to the similar properties (e.g. anatomical and physiological) of its diaphragm to the human diaphragm [Metzger et al., 1985; Mizuno, 1991; Poole et al., 1997]. The diaphragm is comprised of two distinct muscle regions: the costal (ventral, medial, and dorsal) and the crural diaphragm. We elected to focus on the medial costal region. Given its anatomical position and fiber orientation, the medial costal diaphragm sustains the highest proportion of ventilatory work [Poole et al., 1997] and receives the highest mass specific blood flow within the costal diaphragm [Davis et al., 2012; Horn et al., 2020; Horn et al., 2022; Poole et al., 1997; Poole et al., 2000], therefore serving as the primary driver for inspiration. 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 ($23 \pm 2^{\circ}\text{C}$) room with a 12:12-h light-dark cycle with water and rat chow provided ad libitum.

Monocrotaline-induced Pulmonary Hypertension

Pulmonary hypertension (PH) was induced using monocrotaline alkaloid (MCT; 50 mg/kg) administered via a single intraperitoneal injection which has been shown to induce

progressive PH [Gomez-Arroyo et al., 2012; Schulze et al., 2021; Schulze et al., 2022]. MCT (BOC Sciences; Shirley, NY) was dissolved in a solution containing 50% sterile saline and 50% 200-proof ethanol at room temperature and a total of 0.5 mL of fluid was injected into each rat in the experimental group while HC rats received 0.5 mL of vehicle. While maintained on 1-2% isoflurane, injections were given immediately following pre-injection echoes (see *Echocardiography*) with a 27 G needle in the lower right quadrant of the abdomen with care taken to avoid internal organs. Importantly, characteristics of PH (i.e., elevations in pulmonary artery pressures, remodeling of small pulmonary arteries, and right ventricular (RV) hypertrophy) occur 3-4 weeks following injection, prior to the development of overt heart failure [Gomez-Arroyo et al., 2012]. Animals were monitored weekly via echocardiography along with measures of body weight and body condition scoring.

Echocardiography

Transthoracic echocardiography was performed using a commercially available system (Logiq S8; GE Health Care, Milwaukee, WI) with a 13 MHz linear transducer (L4-12t) prior to MCT or saline injection. Subsequent transthoracic echocardiography was performed weekly following injection to monitor disease progression. All comparisons were made between the pre-injection measurement and final measurement taken just prior to experimentation. Animals were initially anaesthetized by inhalation of a 5% isoflurane-O₂ mixture and maintained on $\leq 2.0\%$ isoflurane-O₂ (Butler Health Supply, Dublin, OH). Core temperature was maintained at $\sim 37^{\circ}\text{C}$, measured via rectal thermometer. 2-D and M-mode images of the left ventricle (LV) were obtained from the parasternal short axis window and analyzed for end-systolic/diastolic volumes, stroke volume, and ejection fraction as previously described [Craig et al., 2019]. RV measurements were taken at the level of the aortic valve, just proximal to the pulmonary valve,

in the short axis view. Pulsed Doppler ultrasound was used to assess pulmonary artery ejection time (ET), acceleration time (AT), and peak velocity, and the ratio of AT to ET (AT/ET) was calculated and used as a clinical parameter for the confirmation of PH (AT/ET < 0.3) [Jones et al., 2002]. Upon verification of PH, (average 23 days post-injection) the following experiments were performed.

Surgical Preparation

All surgical procedures were performed using aseptic techniques. Rats were initially anesthetized with a 5% isoflurane-O₂ mixture for 5 min (isoflurane vaporizer; Harvard Apparatus, Cambridge, MA) and subsequently maintained on 3% isoflurane-O₂. Body temperature was maintained at $37 \pm 1^\circ\text{C}$ (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 Inc., 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 (50 mg/ml) 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 over ~30 min. 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.

Diaphragm Contractions

Following carotid and tail artery catheterization, animals were tracheotomized and mechanically ventilated (Kent Scientific PhysioSuite, Torrington, CT). The diaphragm was exposed via laparotomy as previously described [Poole et al., 1995; Geer et al., 2002; Davis et al., 2012]. Following diaphragm exposure, stainless steel electrodes were sutured to the inferior aspect of the right ventral costal (cathode) and the right dorsal costal (anode) diaphragm. Prior to contractions, fluorescent microspheres were infused (described below) to assess baseline diaphragmatic blood flow in the inactive diaphragm. Thereafter, electrically stimulated twitch contractions were induced at 1 Hz (3-6 V, 2-ms pulse duration) with a Grass S88 stimulator (Quincy, MA) for 180s. This stimulation protocol elicits diaphragm contractions at a frequency similar to that of a spontaneously breathing animal [Geer et al., 2002]. Blood flow was measured via fluorescent microspheres (described below) at 180s of stimulation to assess the contracting steady-state diaphragm hyperemia.

Fluorescent Microsphere Injection

The fluorescent microsphere technique, as previously described [Deveci and Eggington, 1999; Musch et al., 1986], was used to quantify tissue blood flow in each experimental group. Fluorescent microspheres were infused at two different time points: 1) baseline (10 min following laparotomy) and 2) at 180s of electrically induced contractions. For each measure, a reference blood sample was taken from the caudal artery catheter, using a Harvard withdrawal pump (model 907, Cambridge, MA) that was initiated 30s prior to microsphere infusion at a withdrawal rate of 0.25 ml/min and $2.0\text{--}2.5 \times 10^5$ fluorescent microspheres (colors: red, scarlet, or 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 kidney or left and right soleus muscle blood flows at each time

point. Following the final microsphere infusion, rats were euthanized with pentobarbital sodium overdose (>50 mg/kg I.A.) followed by cardiac excision. Thereafter, tissues (diaphragm, soleus, intercostal muscles, and kidneys) were harvested, weighed, and placed in 15 ml screw cap polypropylene conical tubes and then placed in -80°C freezer for later blood flow analysis. Using the template previously described [Horn et al., 2020; Horn et al., 2022; Poole et al., 1997], the diaphragm was sectioned into costal (ventral, medial, and dorsal) portions to determine regional distribution of diaphragmatic blood flow, whereas the sum of these portions was used to calculate total costal diaphragm blood flow.

Calculation of Blood Flow and Vascular Conductance

The fluorescent microsphere assay was performed as previously described [Deveci and Eggington, 1999; Horn et al., 2020; Horn et al., 2022]. Tissue blood flow was calculated as follows [Deveci and Eggington, 1999]:

$$\dot{Q} = [(A_t / A_b) \times (s/w)] \times 100$$

where $\dot{Q}$ is blood flow (ml/min/100g), A_t is the individual sample intensity, A_b 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). Vascular conductance was calculated as:

$$VC = \dot{Q} / MAP$$

where VC is vascular conductance (ml/mmHg/min/100g), $\dot{Q}$ is blood flow (ml/min/100g), and MAP is the average mean arterial pressure (mmHg), recorded immediately before and after microsphere infusion. These assays utilized the entire diaphragm and therefore precluded tissue availability for corollary measurements.

Postmortem Measurements

The RV, LV, and lungs were dissected and weighed following euthanasia. RV hypertrophy was measured via the Fulton index: RV weight (mg) / LV + septum (S) weight (mg) [Fulton et al., 1952]. RV hypertrophy was also expressed as RV/body weight.

Data Analysis

Data were analyzed using GraphPad Prism9 (GraphPad Software, San Deigo, CA). Student paired (within animal) and independent two-sample (between HC and MCT) t-tests were utilized to determine differences in echocardiographic and morphometric measurements. Body mass (g), diaphragm mass (mg), and diaphragm:body mass ratio were analyzed using a one-way ANOVA. Tissue blood flows were analyzed with a mixed-effects model. A Grubb's outlier test was performed on all data, and 1 outlier was identified and removed from the respective data subset when detected. Post-hoc analyses were performed using a Tukey's test. All data are presented as means $\pm$ SE. Significance was established at $P < 0.05$.

Results

Morphometric and Echocardiographic Measurements

All MCT rats displayed characteristics consistent with PH including a decreased AT/ET ratio, RV hypertrophy (RV/LV+S), and an elevated lung weight compared to HC animals [Tables 3.1 & 3.2]. Total diaphragm weight did not differ significantly between groups [Table 3.1]. There were no differences in left ventricular ejection fraction, fractional shortening, or stroke volume between HC and MCT rats [Table 3.2; all $P > 0.05$]. Furthermore, heart rate, cardiac output, and cardiac index did not differ between groups [Table 3.4], so we do not believe cardiac factors associated with blood flow to be a limitation to interpretation of the findings herein. MAP was not different between groups, or when comparing baseline to diaphragm

contractions [Table 3.4]. Additionally, there were no differences between groups in regional diaphragm masses, nor in total costal diaphragm mass [all $P>0.05$].

Blood Flow

At baseline (i.e., in the inactive diaphragm), total costal diaphragm blood flow was not different between groups [Figure 3.1]. Despite this, MCT rats had a higher baseline ventral costal blood flow compared to HC and all other costal regions of the diaphragm in MCT and HC rats [Figure 3.3A]. No regional differences were present between costal portions of the diaphragm in HC rats at baseline [Figures 3.2A and 3.3A]. In response to 1 Hz contractions, total costal diaphragm blood flow increased in both HC and MCT animals [Figure 3.1]. In HC rats, 1 Hz contractions elicited the greatest blood flow increase to the medial costal diaphragm [Figure 3.2A]; whereas in MCT animals, the ventral costal portion received the highest baseline and contracting blood flow, and the dorsal costal had the largest increase in blood flow compared to baseline [Figure 3.2B]. Therefore, the hyperemic response of the medial costal diaphragm was greater in HC rats, increasing $55 \pm 3\%$ compared to baseline versus only $44 \pm 4\%$ in MCT rats [Figures 3.2 and 4; $P=0.04$], while the total costal diaphragm hyperemic response was not different between groups (HC, $49 \pm 4\%$ vs. MCT, $41 \pm 12\%$, $P=0.55$). Kidney blood flows fell within the 400-600 ml/min/100g range expected based on previous investigations from our laboratory in all conditions [Horn et al., 2020; Horn et al., 2022; Colburn et al., 2020]. There were no differences between group or condition in blood flow to the kidneys, soleus, or intercostal muscles [Table 3.3; all $P>0.05$].

Vascular Conductance

Total costal diaphragm vascular conductance was not different between HC and MCT rats at baseline (0.38 ± 0.1 vs. 0.46 ± 0.1 ml/mmHg/min/100g; $P=0.5$) or during 1 Hz steady-

state contractions (0.83 ± 0.18 vs. 0.87 ± 0.08 ml/mmHg/min/100g; $P=0.8$). At baseline, vascular conductance was highest in the ventral costal portion of the diaphragm in MCT rats [Figure 3.3C]. During contractions, vascular conductance was highest in the medial costal portion for HC rats, and the ventral costal portion for MCT rats [Figure 3.3D]. Vascular conductance did not differ between groups in the kidneys, soleus, or intercostals (all $P>0.05$).

Discussion

This is the first investigation to our knowledge to demonstrate that PH alters regional diaphragm blood flow distribution and impacts the ability of the diaphragm to augment blood flow with contractile activity. Specifically, PH induced a greater distribution of diaphragm blood flow to the ventral costal diaphragm, a region that in health contributes proportionally less to the work of breathing than the medial and dorsal costal diaphragm. Further, the blunted diaphragm hyperemic response to 1 Hz contractions suggests that PH compromises diaphragm vascular function, potentially via a decreased vasodilatory responsiveness and/or increased tonic vasoconstriction of the diaphragm resistance vasculature. These findings in a female rat model of PH raise the possibility that the pathophysiological consequences of PH extend into the respiratory muscle vasculature and provide a putative mechanism for the diaphragm contractile dysfunction associated with PH.

Monocrotaline-induced PH

This investigation utilized the MCT model of PH at a dose demonstrated to elicit characteristics consistent with the human pathology within 3-4 weeks including medial wall thickening of the pulmonary arteries, elevated right ventricular systolic pressure, pulmonary congestion, and RV hypertrophy [Gomez Arroyo et al., 2012; Nogueira-Ferreria et al., 2015; Schulze et al., 2021; Schulze et al., 2022]. RV hypertrophy is commonly determined using the

Fulton Index (RV/LV+S) or RV/body weight. In this investigation, MCT animals were smaller; however, RV mass was not different, demonstrating a compensatory RV hypertrophy in response to the higher expected afterload. Additionally, PH was confirmed in these rats using clinically established echocardiographic measurements (i.e., AT/ET) [Jones et al., 2002; Kitabatake et al., 1983]. Lower AT/ET has been demonstrated by Kato et al. to be associated with increased RV myocyte cross sectional area and elevated RV/body weight [Kato et al., 2003]. Importantly, this model induces moderate PH, indicating that the alterations in diaphragm blood flow presented herein occur prior to, and independent of, distinct heart failure as indicated by lack of changes in LV ejection fraction [Table 3.2].

Diaphragmatic Blood Flow and Vascular Conductance

Contrary to our hypothesis, bulk diaphragm blood flow [Figure 3.1] and vascular conductance were not different in MCT rats versus HC. Given the heterogenous distribution of blood flow in the diaphragm [Brancatisano et al., 1991; Davis et al., 2012; Poole et al., 1997; Sexton & Poole, 1995; Horn et al., 2020], altered distribution of regional diaphragm blood flow may represent significant changes in the diaphragm vasculature with PH that are masked by only investigating whole diaphragm hemodynamics. In the healthy rodent (i.e., rat), the medial costal diaphragm sustains the greatest proportion of diaphragm inspiratory work and therefore commands the highest blood flow [Poole et al., 1997; Sexton & Poole, 1995]. The diaphragm contractions protocol herein has been used previously in our laboratory and is expected to produce a stimulus akin to spontaneous breathing [Geer et al., 2002; Davis et al., 2012]. Diaphragm blood flow data in the current investigation are consistent with spontaneously breathing animals [Horn et al., 2020; Horn et al., 2022]. In HC animals, medial costal diaphragm blood flow was augmented from baseline to steady-state contractions as expected [Figure 3.2A];

however, in MCT animals there was no significant increase in medial costal diaphragm perfusion during steady-state contractions [Figure 3.2B]. Further, medial costal diaphragm vascular conductance during contractions was lower in MCT rats compared to HC [Figure 3.3D]. These data support that PH impairs the hyperemic response, specifically in the medial costal diaphragm [Figure 3.4]. Lower medial costal vascular conductance and the reduced ability to augment blood flow with contractile activity suggest that PH induces vascular dysfunction in the diaphragm. Such vascular impairments in the diaphragm with PH may be due to a reduced vasodilatory responsiveness, enhanced intrinsic vascular tone, endothelial or vascular smooth muscle signaling mechanisms, changes in neurovascular coupling, and/or structural alterations in the diaphragm resistance vasculature that manifest throughout disease progression, though this remains to be determined.

Previous work suggests that diaphragm contractile dysfunction is predicated by an inability to match oxygen delivery to oxygen demand (i.e., upstream vascular dysfunction) [Supinski, 1988; de Man et al., 2011; Manders et al., 2012]. Our data demonstrate reduced blood flow, and therefore oxygen delivery, to the medial costal diaphragm. Given that blood flow distribution and functional hyperemia is determined by vascular control mechanisms, the blunted medial costal hyperemic response seen with PH is likely due in large part to impaired resistance vessel (i.e., arterioles) vasodilation. Arteriolar dysfunction has been shown across a range of pathological conditions (i.e., aging, hypertension, heart failure, prolonged mechanical ventilation) in several vascular beds [for rev. Vanhoutte et al., 2017; Poole, Behnke & Musch, 2021], including skeletal muscle [Muller-Delp et al., 2002] and the diaphragm [Horn et al., 2019]. Impaired endothelial function has been demonstrated in pulmonary arteries [Mathew et al., 1995, Christou et al., 2018], coronary arterioles [Kajiya et al., 2007], and large systemic

arteries [Wolff et al., 2007; Peled et al., 2008] with PH. However, there is a paucity of data regarding diaphragm vascular function. In PH, dysregulation of the nitric oxide (NO) pathway and production of excess reactive oxygen species (ROS) [Giaid et al., 1995; Humbert et al., 2004; Hampl & Herget, 2000] may act in concert to reduce endothelium-dependent vasodilation [Poole et al., 1997]. Further, the increased levels of endothelin-1 [Rubens et al., 2001] and Angiotensin II [Kumpers et al., 2010] in PH would increase tonic vasoconstriction and blunt NO-mediated vasodilation [Mathew et al., 1995]. The degree to which these markers are upregulated is associated with disease severity. In the present study where animals presented with moderate PH preceding heart failure, the extent of the impact of these vasoconstrictors is not known. Additionally, in the hyperactive diaphragm with PH, $\dot{Q}O_2$ -to- $\dot{V}O_2$ mismatch during increases in metabolic demand could result in local areas of hypoxia (i.e., medial costal) that can promote further ROS generation, thereby damaging the vasculature [Marzetti et al., 2013] and potentially compromising contractile function [Hogan et al., 1992; Hogan et al., 1994; Himori et al., 2017]. Potential structural alterations in the diaphragm vasculature with PH have not been investigated but may play a role in the impaired hyperemic response demonstrated herein. Prolonged arteriolar vasoconstriction can induce vascular remodeling in as little as 4 hours [Martinez Lemus et al., 2004]. Thus, it is possible that the sympathetic hyperactivity, inflammation, and oxidative stress associated with PH could induce vascular remodeling beyond the pulmonary circulation, thereby limiting the necessary increases in medial costal blood flow with contractile activity [Dorfmueller et al., 2003].

Interestingly, ventral costal diaphragm perfusion was higher in MCT rats compared to control both at baseline [Figure 3.3A] and during 1 Hz steady-state contractions [Figure 3.3B], suggesting that PH results in altered blood flow distribution. Considering adequate blood flow

and thus resistance vessel dilation constitute a major determinant of contractile function [Supinski, 1988; Supinski et al., 1993], an inability to meet respiratory muscle oxygen demand would hasten diaphragm fatigue. Therefore, maintenance of a heightened metabolic demand in the chronically hyperactive diaphragm with PH [Laveneziana et al., 2013] may require recruitment of regions that do not contribute as significantly in health. Such a redistribution of blood flow within the diaphragm may actually serve to better match $\dot{Q}O_2$ to $\dot{V}O_2$, although, whether this regional redistribution has a positive or negative effect on local tissue oxygenation, as mentioned above, remains to be determined. Given the physical structure of the ventral costal region of the diaphragm [Poole et al., 1997], greater blood flow to this region at the expense of that to the medial costal region would be expected to reduce overall diaphragmatic contractile force and compromise ventilatory efficiency, thereby serving as a potential mechanism for the diaphragm contractile dysfunction associated with PH.

Absence of Diaphragm Atrophy

Previous investigations in male murine models of PH [Ahn et al., 2013, de Man et al., 2011] demonstrate diaphragm atrophy, however, these studies differ in MCT dosage and time course for PH development (6-8 weeks). In contrast, our MCT rats did not display diaphragm atrophy. While we did not assess cross sectional area of muscle fibers, diaphragm mass and diaphragm mass:body weight were not different between groups [Table 3.1]. The MCT rats in the current investigation are studied prior to development of RV failure in order to assess moderate PH. It is possible that PH-induced diaphragm atrophy is the consequence of late-stage decompensation resulting from the inability to match $\dot{Q}O_2$ to $\dot{V}O_2$, thereby impairing contractile function, generating excess ROS, upregulating markers of atrophy, and inducing eventual diaphragmatic failure.

Experimental Considerations

The present investigation focused on moderate PH preceding RV failure. A longer time course following MCT injection would allow for development of a more severe form of PH that may elicit diaphragm atrophy not seen herein. Future directions may usefully assess $\dot{Q}O_2$ -to- $\dot{V}O_2$ matching in the diaphragm, regional tissue oxygenation, and use histological techniques to determine molecular/structural diaphragmatic or vascular alterations, as well as the time course for development of diaphragm atrophy, blood flow alterations, and contractile dysfunction in this model. Additionally, resting and exercising diaphragm blood flows in the conscious rat could provide insights into increased ventilatory demand, dyspnea, and the exercise intolerance seen with PH. The present investigation used only female rats, thus all findings should be regarded in the context of MCT-induced PH in the female rat. However, available evidence does not support that sex or stage of the estrus cycle impact diaphragm blood flow [Smith et al., 2017]. Furthermore, because females display a higher incidence and greater prevalence of PH, there is a pressing imperative to examine females in PH-focused research [Badesch et al., 2010; Hester et al., 2019]. Future studies may include assessment of the effect of estrogen on the specific diaphragm perfusion measurements herein by inclusion of both male and ovariectomized female rats. A more in-depth discussion of the MCT-PH model in female rats and the necessity to focus on females can be found in Schulze and Musch [Schulze & Musch, 2023]. Finally, the use of MCT to produce PH is a limitation and the possibility of MCT-induced toxicity to the diaphragm should be considered when interpreting these findings. Notwithstanding this concern, MCT is a common and effective PH model, eliciting reliable hallmarks of disease such as pulmonary vascular remodeling and subsequent RV hypertrophy [Gomez Arroyo et al., 2012], and has been widely used to study mechanistically the diaphragm in PH [Ahn et al., 2013; de Man et al., 2011;

Himori et al., 2017; Kanj et al., 1999; Manders et al., 2012]. While off-target effects of MCT on the diaphragm have not been demonstrated, the primary target of MCT given the nature of its metabolism is the pulmonary vasculature and, to our knowledge, there is no evidence that MCT affects skeletal muscle or peripheral vascular function directly. Nonetheless, as with all animal models, application to PH patients should be employed with caution.

Ramifications

The potential consequences of our findings in MCT-induced female rats are that blood flow redistribution toward the ventral costal diaphragm and lack of an increase in medial costal perfusion may be an indication of higher work of a less efficient region of the diaphragm at rest, which would be exacerbated during exercise. This provides a possible mechanism for diaphragm contractile dysfunction as well as dyspnea. Furthermore, the impaired medial costal hyperemic response may also represent an insufficiency to augment blood flow during exercise to this region, which serves as the primary driver of respiration. Importantly, this could contribute to the exercise intolerance reported in PH [Frost et al., 2019]. An inability to comfortably be physically active would act to discourage patients from participating in exercise, which has been shown to mitigate symptoms of PH and improve exercise capacity, muscle function, and quality of life in PH [Arena et al., 2014; Becker et al., 2020; Brown et al., 2017; Chan et al., 2013; de Man et al., 2009; Grunig et al., 2011; Mereles et al., 2006]. The regional diaphragm blood flow redistribution in the present investigation demonstrates a possible impediment to exercise tolerance in PH, and, notably, exercise training and/or respiratory muscle training may serve as a potential therapeutic approach to improve vascular function and normally distribute diaphragm blood flow, mitigating contractile dysfunction in PH.

Conclusions

This investigation reports, for the first time, bulk and regional blood flow and the hyperemic response in the diaphragm from PH rats. Our findings demonstrate that PH alters regional diaphragm blood flow distribution and impairs the ability of the medial costal diaphragm to augment blood flow with contractions. Together, these data provide a putative mechanism for PH-induced diaphragm contractile dysfunction. Given that such alterations occurred prior to development of gross diaphragm atrophy, this suggests the presence of vascular dysfunction within the diaphragm which may precede diaphragm fiber weakness. Whether vascular mechanisms associated with the reduced hyperemic response and redistribution of regional diaphragm blood flow are functional and/or structural warrants further scientific investigation.

Funding: This work was supported in part by the Sustained Momentum for Investigators with Laboratories Established (SMILE) Grants awarded to T.I.M and D.C.P. by Kansas State University College of Veterinary Medicine and the National Institute on Aging 1R15AG078060 awarded to B.J.B and D.C.P. A.G.H and K.M.S. were financially supported by Ruth L. Kirschstein National Research Service Awards 1F31HL167618-01 and 1F31HL170643-01, respectively.

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Table 3.1 Morphometric measurements.

	Healthy Control	Monocrotaline PH
Body weight (g)	297 ± 6	276 ± 7 #
Diaphragm weight (mg)	944 ± 67	938 ± 42
RV weight (mg)	174 ± 10	197 ± 11
LV weight (mg)	681 ± 24	573 ± 19 #
Lung weight (mg)	1188 ± 26	1712 ± 126 #
RV/LV+S (mg/mg)	0.26 ± 0.02	0.37 ± 0.02 #
Diaphragm/body weight (mg/g)	3.18 ± 0.17	3.40 ± 0.08
RV/body weight (mg/g)	0.59 ± 0.03	0.72 ± 0.06 #
LV/body weight (mg/g)	2.30 ± 0.09	2.09 ± 0.08
Lung/body weight (mg/g)	4.0 ± 0.1	6.3 ± 0.5 #

Data are means ± SE; *n*, number of rats; *n*=7 for all; PH, pulmonary hypertension; RV, right ventricle; LV, left ventricle; LV+S, LV + septum; # *P*<0.05 vs. healthy control

Table 3.2 Echocardiographic measurements.

	Healthy Control		Monocrotaline PH	
	Pre	Post	Pre	Post
Acceleration time (ms)	30 ± 2	33 ± 4	29 ± 2	19 ± 1 #
Ejection time (ms)	84 ± 2	85 ± 2	82 ± 3	81 ± 3
AT/ET	0.36 ± 0.02	0.39 ± 0.04	0.35 ± 0.02	0.23 ± 0.01 #
Ejection fraction (%)	84 ± 2	86 ± 3	85 ± 2	86 ± 4
Stroke volume (ml)	0.48 ± 0.05	0.53 ± 0.02	0.53 ± 0.04	0.52 ± 0.05
Fractional shortening (%)	48 ± 3	52 ± 4	50 ± 2	53 ± 5

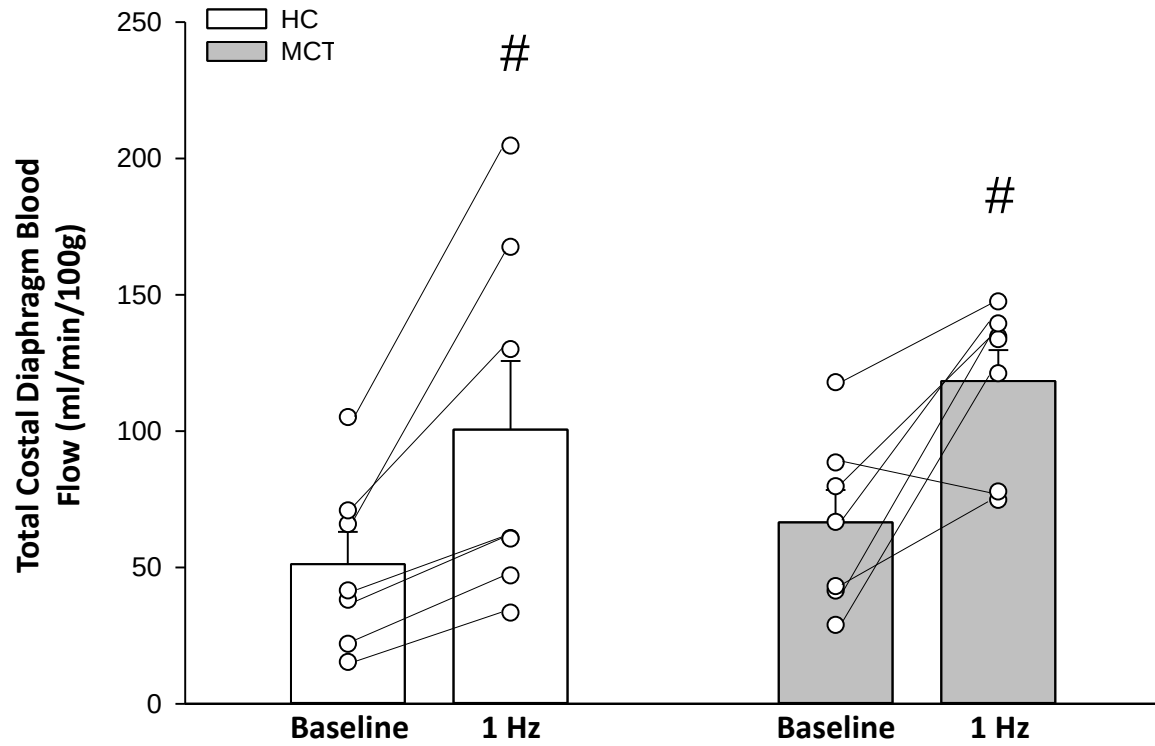
Data are means ± SE; *n*, number of rats; *n*=7 for all; PH, pulmonary hypertension; AT, acceleration time; ET, ejection time; # *P*<0.05 vs. all other conditions

Table 3.3 Blood flow.

	Healthy Control		Monocrotaline PH	
	Baseline	1 Hz	Baseline	1 Hz
Kidney	431 ± 46	457 ± 56	505 ± 70	594 ± 94
Soleus	48 ± 14	45 ± 15	46 ± 11	32 ± 7
Intercostal	38 ± 16	31 ± 8	21 ± 4	29 ± 10

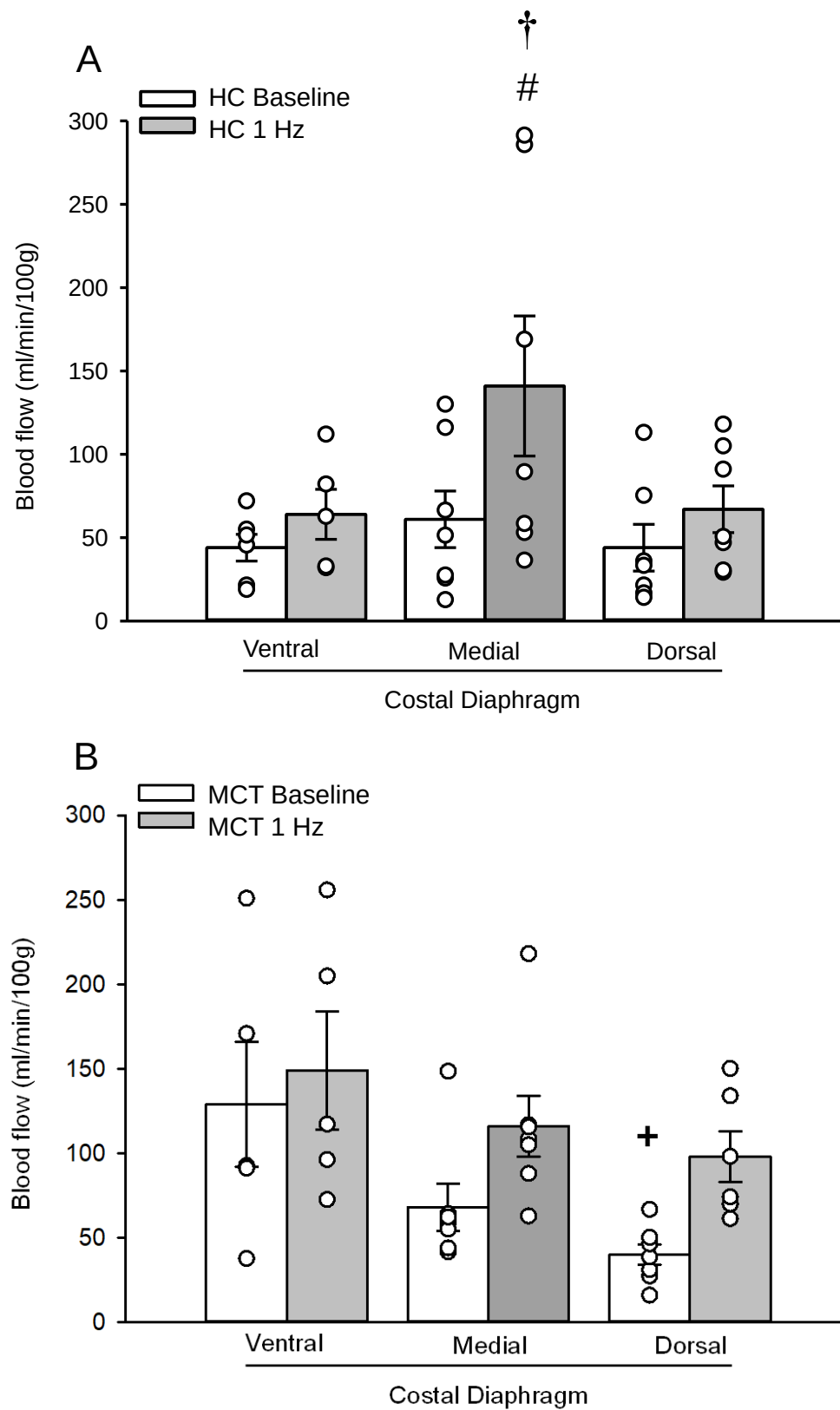
Data are means ± SE; Units are ml/min/100g; n, number of rats; n=7 for all; PH, pulmonary hypertension

Figure 3.1 Total costal diaphragm blood flow.



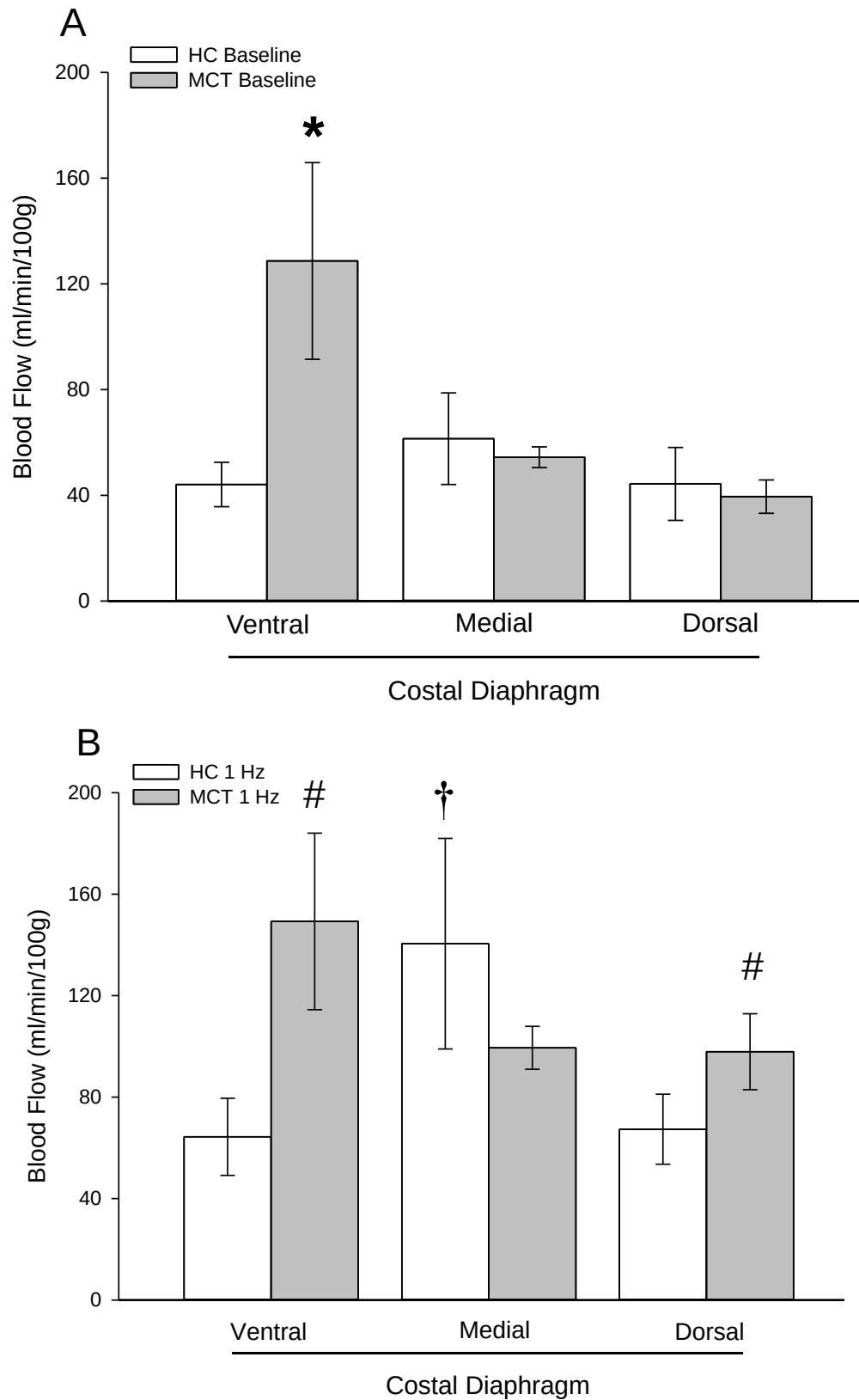
Total costal diaphragm blood flow (ml/min/100 g) in healthy control (HC; n = 7) and monocrotaline-induced pulmonary hypertension (MCT; n = 7) animals at baseline and during steady-state 1 Hz contractions. #Significant ($p < 0.05$) versus baseline

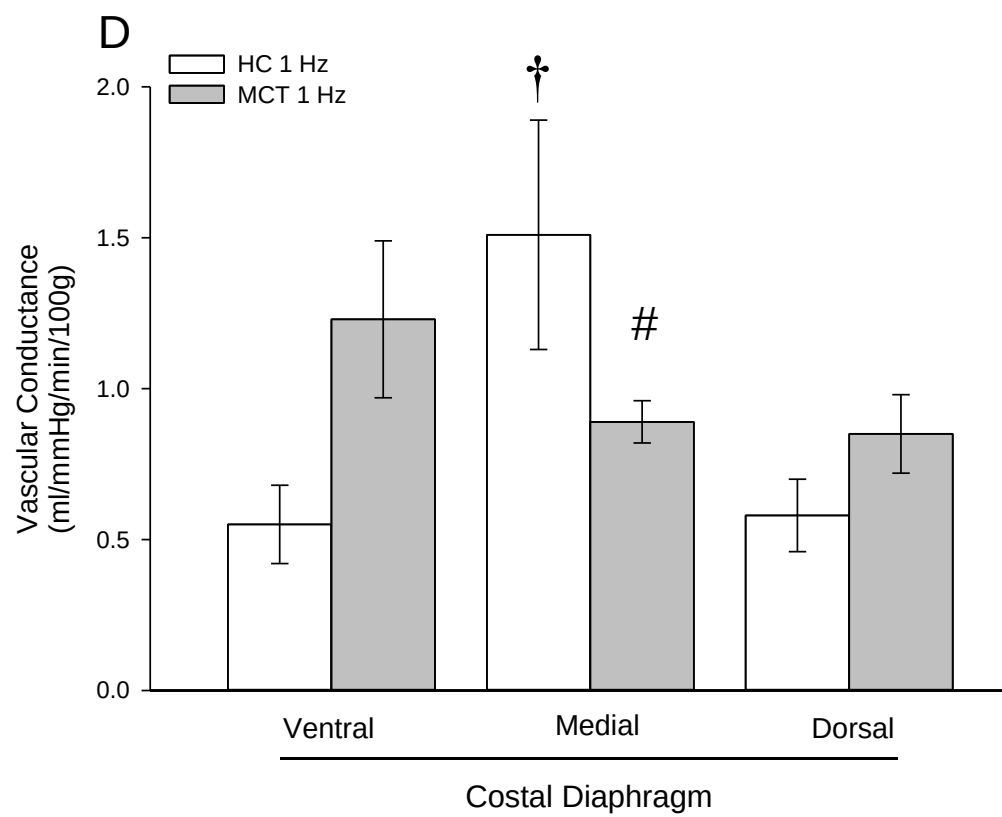
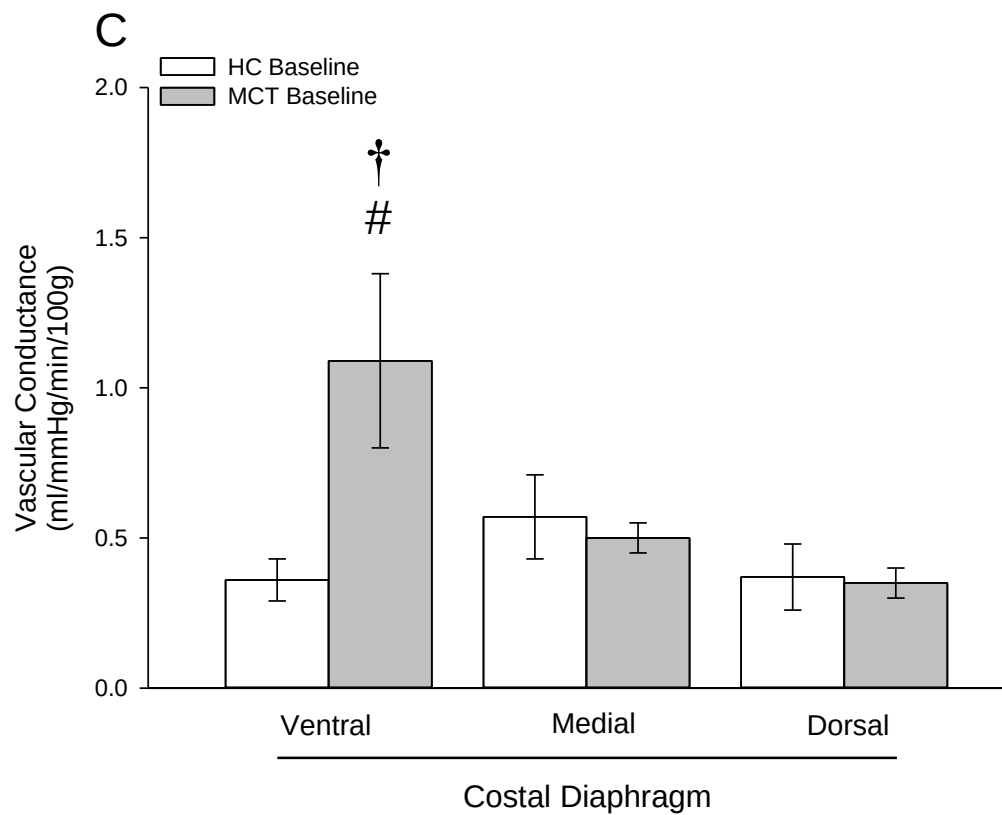
Figure 3.2 Regional diaphragm blood flow at rest versus contractions.



Regional diaphragm blood flow (ml/min/100 g) at baseline and during steady-state 1 Hz contractions in healthy control (HC; n = 7) (A) and monocrotaline-induced pulmonary hypertension (MCT; n = 7) (B) animals. #Significant ($p < 0.05$) versus baseline within diaphragm region. †Significant ($p < 0.05$) versus other diaphragm regions at 1 Hz. +Significant ($p < 0.05$) versus ventral costal baseline.

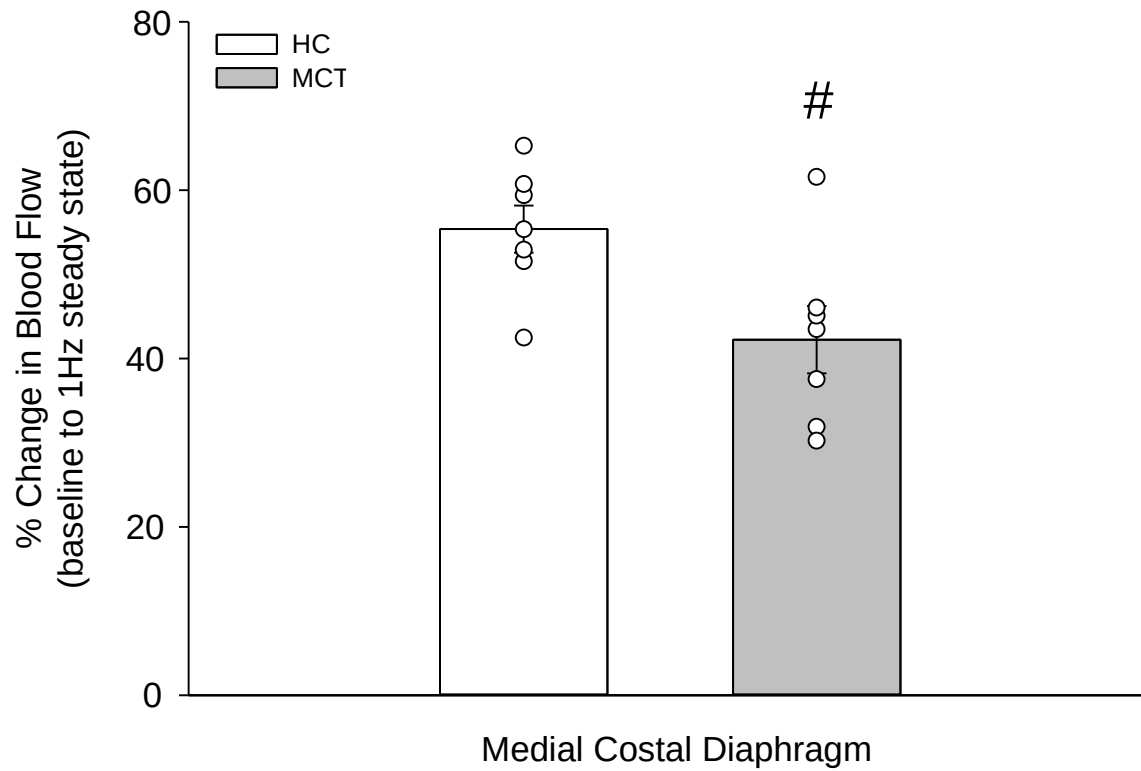
Figure 3.3 Regional diaphragm blood flow and vascular conductance.





Regional diaphragm blood flow at baseline (A) and during steady-state 1 Hz contractions (B) and vascular conductance (ml/mmHg/min/100 g) at baseline (C) and during steady-state 1 Hz contractions (D) in healthy control (HC; n = 7) and monocrotaline-induced pulmonary hypertension (MCT; n = 7) animals. *Significant ($p < 0.05$) versus all other bars (Figure 3.3A). #Significant ($p < 0.05$) versus HC within diaphragm region. †Significant ($p < 0.05$) versus other diaphragm regions within group (HC or MCT).

Figure 3.4 Medial costal hyperemic response.



The percent change in medial costal diaphragm blood flow from baseline to steady-state 1 Hz contractions in healthy control (HC; n = 7) and monocrotaline-induced pulmonary hypertension (MCT; n = 7) animals. #Significant ($p < 0.05$) versus HC.

Chapter 4 - Pulmonary Hypertension Impairs Vasomotor Function in Rat Diaphragm Arterioles

Abstract

Pulmonary hypertension (PH) is a chronic, progressive condition in which respiratory muscle dysfunction is a primary contributor to exercise intolerance and dyspnea in patients. Contractile function, blood flow distribution, and the hyperemic response are altered in the diaphragm with PH, and we sought to determine whether this may be attributed, in part, to impaired vasoreactivity of the resistance vasculature. We hypothesized that there would be blunted endothelium-dependent vasodilation and impaired myogenic responsiveness in arterioles from the diaphragm of PH rats. Female Sprague-Dawley rats were randomized into healthy control (HC, n=9) and monocrotaline (MCT, n=9)-induced PH rats. Endothelium-dependent and -independent vasodilation and myogenic responses were assessed in first-order arterioles (1As) from the medial costal diaphragm in vitro. There was a significant reduction in endothelium-dependent (via acetylcholine; HC, $78 \pm 15\%$ vs. MCT, $47 \pm 17\%$; $P < 0.05$) and -independent (via sodium nitroprusside; HC, $89 \pm 10\%$ vs. MCT, $66 \pm 10\%$; $P < 0.05$) vasodilation in 1As from MCT rats. MCT-induced PH also diminished myogenic constriction ($P < 0.05$) but did not alter passive pressure responses. The diaphragmatic weakness, impaired hyperemia, and blood flow redistribution associated with PH may be due, in part, to diaphragm vascular dysfunction and thus compromised oxygen delivery which occurs through both endothelium-dependent and -independent mechanisms

Introduction

Pulmonary arterial hypertension (PH) is a vascular disease wherein pulmonary arterial remodeling elevates pulmonary arterial pressures, impairs gas exchange, and eventually leads to right ventricular failure. Exercise intolerance and dyspnea are key characteristics of PH which hinder the quality of life for PH patients [Halank et al., 2013]. Whilst the mechanisms involved in dyspnea and exercise intolerance with PH are multifaceted, diaphragm dysfunction serves as one possible contributor that has received scant attention in PH. Inefficient gas exchange demands greater work of the respiratory muscles to maintain alveolar ventilation, resulting in chronic tachypnea at rest and during exercise in PH patients [Sun et al., 2001; 2003]. However, evidence of diaphragmatic impairments such as reduced sniff velocity [Meyer et al., 2005], diaphragm fiber atrophy, and contractile weakness [Ahn et al., 2013; de Man et al., 2011] have been demonstrated in PH patients and animal models. Thus, the diaphragm is weakened in PH, while also attempting to maintain heightened ventilatory work, predicated respiratory muscle failure.

The sources of diaphragmatic abnormalities in PH are complex and remain incompletely understood. Signaling mechanisms have been suggested to play a role in the reduced contractile function and fiber atrophy in the PH diaphragm [de Man et al., 2011; Kosmas et al., 2022], although these findings are inconsistent [Ahn et al., 2013, Manders et al., 2012]. Crucially, a key facet of muscle function is oxygen delivery and the ability to augment or match blood flow in response to an increased metabolic demand. Recently, our lab has demonstrated that monocrotaline (MCT)-induced PH impairs the medial costal diaphragmatic hyperemic response to contractions [Schulze et al., 2023]. Considering that the medial costal diaphragm commands the greatest blood flow compared to other regions which suggests that it contributes appreciably

to the work of breathing [Poole et al., 1997; Sexton & Poole 1995], a reduced hyperemic response in this region represents an unrecognized possible source for the diaphragm contractile impairments and dyspnea present with PH that may be the result of vascular (i.e., arteriolar) dysfunction.

Endothelial dysfunction is present in both the pulmonary and brachial arteries in PH, and is attributed, in part, to disruption of the nitric oxide (NO) pathway, increased production of reactive oxygen species (ROS), and inflammation [Peled et al., 2008; Wolff et al., 2007; Mathew et al., 1995; Nakazawa et al., 1999]. This physiological milieu also is known to contribute to impaired endothelium-dependent vasodilation in hindlimb muscle arterioles of aged animals [Muller-Delp et al., 2002b] and diaphragm arterioles following prolonged inactivity induced via mechanical ventilation [Horn et al., 2019]. Similar perturbations may exist in the hyperactive diaphragm with PH, however, diaphragm vascular function has not yet been investigated. We hypothesize that first-order arterioles (1A) from the medial costal diaphragm of monocrotaline MCT-induced PH rats will demonstrate 1) reduced endothelium-dependent vasodilation with preserved endothelium-independent vasodilation, 2) an impaired myogenic response, and 3) no differences in passive pressure responses compared to healthy controls. The findings of this investigation will provide insights to the mechanistic bases for diaphragmatic atrophy and contractile dysfunction in PH.

Methods

Female Sprague-Dawley rats (4-5 months old, ~275 g, n = 18) were obtained from Charles River Laboratories (Boston, MA, USA) for this investigation. Animals were randomized into healthy control (HC, n = 9) and monocrotaline (MCT)-induced PH animals (MCT, n = 9). The Sprague-Dawley rat was chosen due to the similar properties (e.g. anatomical and

physiological) of its diaphragm to the human diaphragm [Metzger et al., 1985; Mizuno, 1991; Poole et al., 1997]. The diaphragm is comprised of two distinct muscle regions: the costal (ventral, medial, and dorsal) and the crural diaphragm. We elected to study medial costal first-order arterioles, as the medial costal diaphragm receives the highest mass specific blood flow within the costal diaphragm in health [Davis et al., 2012; Horn et al., 2020; Sexton & Poole 1995; Poole et al., 2000], and impairments in diaphragm microcirculatory function would be expected to be present in the medial costal portion [Schulze et al., 2023]. All procedures were approved by the Kansas State University Institutional Animal Care and Use Committee and complied with the ARRIVE guidelines and National Institutes of Health Guide for the Care and Use of Laboratory Animals (#4573; Approved July 2023). Upon arrival, animals were housed and maintained in a temperature-controlled ($23 \pm 2^{\circ}\text{C}$) room with a 12:12-h light-dark cycle with water and rat chow provided ad libitum.

Monocrotaline-induced Pulmonary Hypertension

Monocrotaline alkaloid (MCT; 50 mg/kg) was administered via a single intraperitoneal injection which has been shown to induce progressive PH [Gomez-Arroyo et al., 2012; Schulze et al., 2021; 2022; 2023]. MCT (BOC Sciences; Shirley, NY) was dissolved in a solution containing 50% saline and 50% 200-proof ethanol at room temperature and a total of 0.5 mL of fluid was injected into each rat in the experimental group while HC rats received 0.5 mL of vehicle. While under anesthesia (2% isoflurane), injections were performed with a 27 G needle in the lower right quadrant of the abdomen immediately following pre-injection echoes (see Echocardiography). Importantly, characteristics of PH (i.e., elevations in pulmonary artery pressures, remodeling of small pulmonary arteries, and right ventricular (RV) hypertrophy) occur 3-4 weeks following injection [Gomez-Arroyo et al., 2012]. Our laboratory has previously

shown that this model elicits gross RV hypertrophy in both male and female Sprague-Dawley rats in this time frame [Schulze et al., 2021; 2022; 2023]. Animals were monitored weekly via echocardiography along with documentation of body weight and behavioral changes.

Echocardiography

Transthoracic echocardiography was performed using a commercially available system (Logiq S8; GE Health Care, Milwaukee, WI) with a 13 MHz linear transducer (L4-12t) prior to MCT or saline injection, and then weekly to monitor disease progression as previously described [Schulze 2021; 2022; 2023]. All comparisons were made between the pre-injection measurements and final measurements taken just prior to experimentation. Animals were initially anaesthetized by inhalation of a 5% isoflurane-O₂ mixture and maintained on $\leq 2.0\%$ isoflurane-O₂ mixture (Butler Health Supply, Dublin, OH). Core temperature was maintained via heating pad at $\sim 37^{\circ}\text{C}$, measured via rectal thermometer. 2-D and M-mode images of the left ventricle (LV) were obtained from the parasternal short axis window and analyzed for end-systolic/diastolic volumes, stroke volume, and ejection fraction as previously described [Craig et al., 2019]. RV measurements were taken at the level of the aortic valve, just proximal to the pulmonary valve, in the short axis view. Pulsed Doppler ultrasound was used to assess pulmonary artery ejection time (ET), acceleration time (AT), and peak velocity. The ratio of AT to ET (AT/ET) was calculated, which serves as an established indicator of RV function and is used as a clinical parameter for the confirmation of PH (AT/ET < 0.30) [Jones et al., 2002]. Upon verification of PH (24 ± 3 days post-MCT injection), rats were euthanized under a deep plane of isoflurane anesthesia via pentobarbital sodium overdose (>100 mg/kg) and the following experiments were performed.

Isolated Microvessel Technique

To determine vasomotor control, resistance arterioles (<200 μm intraluminal diameter) from the medial costal diaphragm were isolated and studied in vitro to remove potentially confounding metabolic, humoral, and neural influences. 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 [Aaker and Laughlin, 2002a; 2002b]. 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) and video caliper (Colorado Video 307A) for 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 exhibited leaks were discarded. In arterioles that were free from leaks, the poly(methyl methacrylate) chamber was connected to a water circulating heater and vessels were warmed to 37°C and allowed to develop initial spontaneous tone during a 60 min equilibration period. Endothelium-dependent (i.e exposure to acetylcholine) and -independent (sodium nitroprusside, SNP), and myogenic (active and passive) responses were assessed in medial costal diaphragm 1As, as described below.

Assessment of Vasomotor Function

Spontaneous tone development

Medial costal 1As were equilibrated in warm PSS at 37°C for 60 min (refreshed every 20 min) to allow for the development of spontaneous tone [Table 4.2]. Spontaneous tone development was expressed as the percent constriction relative to maximal diameter and was calculated using the formula [Muller-Delp et al., 2002a; 2002b]:

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

where ID_{max} represents the maximal inner diameter, and ID_{b} represents the baseline inner diameter following the 60 min equilibration period and before the addition of any drug.

Acetylcholine-mediated vasodilation

Acetylcholine (ACh) mediates smooth muscle relaxation indirectly by binding to endothelial cell muscarinic receptors (M_2 , M_3) and stimulates the release of endothelium-derived relaxing factor(s), primarily nitric oxide (NO) [Vanhoutte et al., 2017]. Upon displaying a steady level of spontaneous tone, vasodilatory responses to increasing concentrations of ACh (10^{-9} to 10^{-4} M) administered at 2 min intervals and steady-state diameters were recorded at the end of each 2 min interval, as previously described [Horn et al., 2019]. In subsets of animals, the contribution of endothelial nitric oxide synthase (eNOS) and cyclooxygenase (COX) in medial costal 1As was assessed by performing the same ACh concentration-response as above following 20 min incubation with 10^{-5} M of the NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME). Thereafter, in a subset of animals used to L-NAME responses, the same vessel underwent a 20-min incubation period with 10^{-5} M L-NAME plus 10^{-5} M indomethacin (INDO). Time control experiments were performed in a cohort of animals which verified that there is no desensitization to ACh throughout this protocol.

Sodium nitroprusside-mediated vasodilation

Sodium nitroprusside (SNP) is a potent vasodilatory agent that acts directly on smooth muscle through NO-cGMP activation to elicit vasodilation. To determine endothelial-independent function of medial costal diaphragm 1As, concentration responses to the exogenous NO donor SNP (10^{-9} to 10^{-4} M) were assessed.

Vasodilatory responses were recorded as actual diameters and represented as percentage of maximal dilation. Dilation (%) of diaphragm arterioles was calculated according to the formula [Muller-Delp et al., 2002b; Horn et al., 2019]:

$$\text{Dilation (\%)} = (D_s - D_b) / (D_m - D_b)$$

where D_m is the maximal inner diameter recorded at 90 cmH₂O in calcium-free PSS, D_s is the steady-state inner diameter recorded after each addition of the drug, and D_b is the initial baseline inner diameter recorded immediately before the first addition of the vasodilatory agent. EC_{50} and EC_{25} for vasodilatory responses were calculated with a linear fit model using the two points flanking the estimated half- and quarter-maximal dilation.

Active and passive myogenic responses

Active myogenic responses were assessed by increasing intraluminal pressure in a stepwise fashion in 20-cmH₂O increments from 0 to 140 cmH₂O. Diameter was recorded for 3 min at each pressure step and these pressure changes occurred in the absence of flow. Thereafter, to assess the passive response, the vessels were incubated in calcium-free PSS for 30 min (bath changed every 10 min) at 37 °C and 90 cmH₂O. Following equilibration, intraluminal pressure was again increased in 20 cmH₂O increments from 0 to 140 cmH₂O and diameter was recorded for 3 min at each pressure step in the absence of flow, as previously described [Horn et al., 2021].

After completion of the final passive pressure-response, vessels were incubated for 30 min in calcium-free PSS (bath change every 10 min). Thereafter, vessels were exposed to one 40 μ l dose of SNP [10^{-4} M], and maximal diameters and wall thickness were recorded.

Active myogenic and passive-pressure diameter responses to incremental changes in intraluminal pressure from 0 to 140 cmH₂O were normalized according to [Muller-Delp et al., 2002a; Horn et al., 2019]:

$$\text{Normalized diameter} = (\text{IDs} / \text{ID}_{\text{max}})$$

where IDs is the steady-state inner diameter measured after each incremental 20 cmH₂O pressure change.

Data Analysis

Vessel characteristics, morphometric measurements, and EC₅₀, were analyzed via independent two-sample t-test. Vasomotor responses and echocardiographic measures were evaluated by two-way ANOVA to detect main effects and interactions between (HC vs. MCT) and within (pre/post, pressure, inhibitor treatment, etc.) groups. Post-hoc analyses were performed using a Tukey's test. All data are presented as means $\pm$ SD. Significance was established at $P < 0.05$.

Results

Morphometric, Echocardiographic, Protein, and Vessel Characteristic Measurements

All MCT rats displayed reduced AT/ET detected via echocardiography compared to pre-injection and healthy controls, a primary diagnostic criterion for indicating PH development [Table 4.1; $P < 0.05$]. Echocardiographic measures of LV function (stroke volume, ejection fraction, fractional shortening) did not differ between HC and MCT groups at pre-injection or final echocardiograms [Table 4.1; $P > 0.05$]. Diaphragm mass was not different between HC and

MCT animals [Table 4.2; $P = 0.97$]. No differences in spontaneous tone development, maximal diameter, maximal wall thickness, or wall-to-lumen ratio were detected between the diaphragm resistance vessels from HC versus MCT rats [Table 4.2; all $P > 0.05$].

Endothelium-Dependent Vasodilatory Responses

In MCT rats, there was a ~40% reduction in endothelium-dependent vasodilation to ACh compared to diaphragm arterioles from healthy animals [Figure 4.1; $P < 0.05$]. In the presence of L-NAME, the response to ACh was significantly diminished in both groups, abolishing group differences to ACh, although the reduction was greater in the HC group versus the MCT group [Figure 4.1]. Compared with L-NAME alone, combining NOS and COX blockade with L-NAME and indomethacin, respectively, further reduced responses to ACh in both groups, with no differences between groups in either the reduction from L-NAME alone, or the vasodilatory response curves [Figure 4.1].

Endothelium-Independent Vasodilatory Responses

MCT rats demonstrated a blunted endothelium-independent dilation to the exogenous NO donor SNP compared with responses of diaphragm arterioles from the HC group [Figure 4.2]. Maximal dilation to SNP was reduced by 26% in diaphragm arterioles from MCT rats. In the HC group, there were no differences in dilation in response to ACh and SNP [Figure 4.3A]; however, with MCT, there was a 29% reduction in the maximal dilation to ACh compared to SNP [Figure 4.3B]. No differences were detected in arteriolar sensitivity (EC_{50}) to ACh in any condition or SNP between HC and MCT rats ($P > 0.10$ for all). Despite this, EC_{25} to ACh was greater in MCT rats representing the blunted vasodilation at lower concentrations of ACh [Table 4.3].

Active and Passive Myogenic Tone

There were no differences in passive pressure responses between MCT and HC rats [Figure 4.4]. Arterioles from both HC and MCT rats displayed active myogenic constriction with stepwise increases in intraluminal pressure from 20-140 cmH₂O. The active response was significantly diminished in the MCT rats versus the HC [Figure 4.4]. Therefore, myogenic constriction was less in diaphragm arterioles from MCT rats compared to HC.

Discussion

This is the first investigation to demonstrate substantial decrements in diaphragm vasomotor function in a rat model of pulmonary hypertension. Specifically, both endothelium-dependent and -independent vasodilation, and myogenic regulation of medial costal diaphragm arterioles were impaired in MCT rats compared to healthy controls. Interestingly, in the presence of L-NAME, group differences in endothelium-dependent vasodilation were abolished, implicating dysregulation of the nitric oxide pathway in the PH-induced diaphragm vascular dysfunction demonstrated herein. Furthermore, the reduction in endothelium-independent vasodilation in MCT rats suggests that there are also vascular smooth muscle impairments, contrary to our hypothesis. These findings demonstrate that the vascular pathological effects of PH extend beyond the pulmonary circulation, and provide a putative mechanism for the diaphragm contractile dysfunction associated with PH.

Vasodilatory Function

Previous work has suggested that diaphragm contractile dysfunction with PH is predicated by an inability to match oxygen delivery to oxygen demand (i.e., upstream arteriolar dysfunction) [de Man et al., 2011; Manders et al., 2012]. Indeed, the results of the present investigation support the presence of both endothelial and vascular smooth muscle dysfunction in diaphragm arterioles.

Vascular Dysfunction in PH

Fundamentally, the pathogenesis of PH includes pulmonary vascular remodeling and blunted vasoreactivity in the pulmonary arteries [Christou & Khalil, 2022]. A number of studies report impaired endothelium-dependent and -independent dilation in isolated pulmonary arteries and arterial rings from rats with PH which is commonly suggested to result from nitric oxide pathway dysfunction [Mathew et al., 1995; Mam et al., 2010; Christou et al., 2018; Nakazawa et al., 1999], but reduced vascular function has also been demonstrated in other vascular beds in PH [Budhiraja et al., 2004]. Wolff et al. (2007) were the first, to our knowledge, to report endothelial dysfunction beyond the pulmonary circulation in PH patients. They concluded that flow-mediated dilation of the brachial artery was reduced with PH [Wolff et al., 2007]; while in MCT rats, it has been demonstrated that endothelium-dependent dilation is blunted in RV coronary arterioles [Kajiya et al., 2007]. Importantly, the present investigation is the first to demonstrate diaphragm microvascular dysfunction with MCT-induced PH. Specifically, the isolated microvessel technique used herein assesses vasoreactivity in the absence of neuronal or humoral influence; thus, these findings demonstrate that diaphragm arteriolar dysfunction, intrinsic to the vasculature, may play a critical role in maintaining diaphragm muscle oxygenation and contractility.

Endothelium-Dependent Vasodilation

Oxidative stress and inflammation are highly implicated in the pathogenesis of PH [Humbert et al., 1995; 2004; Budhiraja et al., 2004] and may serve as interrelated mechanisms playing a role in the diaphragm endothelial dysfunction herein, which appears to be largely mediated by alterations in NO bioavailability and/or eNOS function (Figure 4.1). Inflammatory markers (i.e., interleukin-6, tumor necrosis factor- α) can reduce NO bioavailability, increase

expression of superoxide anion, and uncouple eNOS, [Alonso et al., 1997; Griendling & Alexander, 1997; McIntyre et al., 1999], all of which have been reported in PH [Klinger 2013; Giaid & Saleh, 1995] and would further promote ROS formation. These ROS can scavenge NO [Zhang et al., 2011] and induce vascular damage [Muller-Delp et al., 2012]. This environment is a hallmark of conditions such as heart failure, diabetes, systemic hypertension, and aging, where endothelial dysfunction has been reported in soleus and coronary arterioles [Vanhoutte et al., 2017; Muller-Delp 2002b; Csiszar et al., 2002; Kang et al., 2009a; Koller & Huang, 1994]. Furthermore, Horn and colleagues have demonstrated that mechanical ventilation induces rapid diaphragm arteriolar dysfunction [Horn et al., 2019], attributed in part to oxidative stress and impaired NO signaling, which contributes to a compromised hyperemic response to diaphragm contractions and low microvascular oxygen pressure (PO_2) [Davis et al., 2012]. Thus, impaired NO bioavailability, ROS, and inflammation represent common underlying mechanisms that likely contribute to the blunted endothelium-dependent vasodilation herein.

Endothelium-Independent Vasodilation

Endothelial dysfunction alone does not completely explain the diminished vasodilatory responses of diaphragm arterioles in this investigation. Beyond the endothelium, vascular smooth muscle dysfunction could be related to downstream NO pathway dysfunction or vascular remodeling. It is also possible that exogenously donated NO (SNP) is scavenged within the smooth muscle cell. The NO-sGC-cGMP cascade, requisite for vasodilation, is dysregulated in PH pulmonary vessels, and thus commonly targeted by PH therapies [Christou & Khalil, 2022]. The presence of oxidized (i.e., inactive) sGC, increased phosphodiesterase activity, and altered Ca^{2+} handling in peripheral arterioles, akin to that seen in the pulmonary vasculature, could provide a mechanism for the impaired endothelium-independent vasodilatory function seen in

the diaphragm [Christou & Khalil, 2022; Klinger et al., 2013]. Another possibility may be that elevated sympathetic activity [Velez Roa et al., 2004] or increases in circulating vasoconstrictors such as angiotensin II, [Pezzuto et al., 2015] endothelin-1 [Giaid et al., 1993; Rubens et al., 2001], and thromboxane-A₂ [Christman et al., 1992] in PH could increase tonic vasoconstriction, opposing vasodilation. As the vessels herein were investigated ex vivo, circulating factors and neuronal activity would only impact vascular tone in the current study via chronic adaptations of the vasculature to these factors.

Vasomotor Autoregulation

The myogenic response of resistance vasculature is essential to the control of muscle blood flow, which, in the present investigation, was markedly reduced in diaphragm arterioles from MCT rats. This finding is consistent with the blunted myogenic responsiveness of skeletal muscle arterioles from aged rats [Ghosh et al., 2015; Kang et al., 2009b; Muller-Delp et al., 2002a] but, to our knowledge, the myogenic response in diaphragm arterioles in PH has not previously been assessed. It has been demonstrated that pulmonary arteries from PH rats constrict less to KCl and phenylephrine than pulmonary arteries from healthy controls [Christou et al., 2018; Mam et al., 2010]. However, no differences in vasoconstriction from non-pulmonary vessels (i.e., aorta or mesentery) were noted in that study. This might indicate heterogeneity and differential regulation across vascular beds, and/or that impairments in vasoreactivity occur in the diaphragm arterioles prior to that in conduit arteries or other tissue vasculature. Additionally, reduced Ca²⁺ sensitivity has been demonstrated in diaphragm muscle fibers in PH, lowering the force generated for a given level of Ca²⁺ [Manders et al., 2012]. If also present in diaphragm vascular smooth muscle cells, this phenomenon would help explain the blunted vasoconstriction at a given pressure. Future studies are needed to determine the mechanisms responsible for the

impaired myogenic responses found herein by assessing diaphragm vascular responses to vasoconstrictors and/or using blockade of pressure-sensitive channels implicated in the myogenic response. Nonetheless, inability of the diaphragm vasculature to autoregulate as demonstrated in the present investigation would impair the inherent ability of the medial costal diaphragm to control bulk perfusion and its distribution, which could contribute to the diaphragm blood flow redistribution recently described in MCT-induced PH [Schulze et al., 2023].

Initially, the blunted endothelium-independent vasodilation to an exogenous NO donor could be due, in part, to altered structural properties (e.g., increased stiffness) of the diaphragm arterioles with PH. However, no differences were detected in passive pressure responses between HC and MCT rats, which would have indicated gross structural changes in the arterioles [Muller-Delp et al., 2002a; Delp et al., 2000; Davis & Hill, 1999]. Despite no impact on gross passive responses, it is possible that alterations in the stress-stretch relationship or structural and material properties of the vasculature, such as changes in the composition of the vascular wall, are present [Muller-Delp et al., 2018]. Christou and colleagues [Christou et al., 2018] have demonstrated that pulmonary arteries from PH rats display a shift from a contractile to a more proliferative phenotype with a loss of contractile proteins, which, if present in the diaphragm vasculature with PH, could help explain the findings herein. Prolonged vasoconstriction can induce conformational changes such as vascular smooth muscle cell repositioning within the vascular wall [Martinez Lemus et al., 2008] potentially mediated by matrix metalloproteinase activation [Martinez Lemus et al., 2011]. Matrix metalloproteinases [Chelladurai et al., 2012], circulating inflammatory cytokines [Humbert et al., 1995], altered shear stress, and chronically elevated sympathetic activity [Velez Roa et al., 2004] in PH are potential mechanisms that could induce

vascular remodeling in the diaphragm vasculature, and contribute to the reduced myogenic tone development documented herein and warrants further investigation.

Ramifications

Our findings support the presence of vascular dysfunction in the medial costal diaphragm, the region which, in health, is positioned to sustain the greatest proportion of diaphragm inspiratory work and, consistent with this notion, commands the highest blood flow [Poole et al., 1997; Sexton & Poole, 1995]. Thus, impaired vasodilatory and myogenic responses in the medial costal arterioles would explain the blunted hyperemic response to this region and the regional redistribution recently reported in the diaphragm from MCT-induced PH rats [Schulze et al., 2023]. Further, adequate blood flow, and thus resistance vessel function, is a major determinant of diaphragm contractile function. Specifically, reductions in diaphragm blood flow promote diaphragmatic fatigue [Supinski et al., 1988]. Therefore, these findings imply that reduced vasodilatory function would impact regional oxygen delivery ($\dot{Q}O_2$)-to-utilization ($\dot{V}O_2$) matching, lowering muscle PO_2 thereby predicating contractile dysfunction [Hogan et al., 1992] and diaphragm-specific weakness in PH [de Man et al., 2011].

Experimental Considerations

The current investigation used only female rats. The greater prevalence of PH in females compels the study of female rats to understand potential consequences of disease in this typically understudied population. Given the effect of sex on PH development and outcomes, we plan to assess sexual dimorphism in diaphragm vascular function using this PH model in the future. To our knowledge, there is no evidence for sex differences in diaphragm blood flow and vascular function, although the role of estrogen in cardiovascular disease cannot be overlooked [Smith et al., 2017]. We utilized the MCT model of PH which produces characteristics consistent with the

human pathology within ~3 weeks including medial wall thickening of the pulmonary arteries, elevated right ventricular systolic pressure, and RV hypertrophy [Gomez Arroyo et al., 2012; Schulze et al., 2021; Schulze et al., 2022; Schulze et al., 2023]. Off-target effects of MCT on the diaphragm have not been determined; however, the metabolism of MCT allows it to primarily affect the pulmonary vasculature and, to our knowledge, MCT does not affect peripheral vascular function directly. Furthermore, future directions might usefully assess vascular function in PH across all regions of the diaphragm and in other respiratory muscles. Importantly, this model induces moderate PH preceding cardiac failure as indicated by lack of changes in LV ejection fraction. Therefore, the vascular dysfunction presented herein occurs prior to, and independent of, distinct heart failure. Contrary to previous investigations [Ahn et al., 2013; de Man et al., 2011], gross diaphragm atrophy (i.e., reduced diaphragm mass) was not present in this study. This contrast may be due to the time course for disease progression and model used, as moderate-severity MCT-induced PH rats were used herein. A longer time interval following MCT injection will allow for development of a more severe form of PH that may elicit diaphragm atrophy not seen herein.

Conclusions

The key original findings of this investigation are that MCT-induced PH reduces endothelium-dependent and -independent vasodilatory function in the medial costal first-order diaphragm arterioles and that blunted endothelium-dependent vasodilation is mediated, in large part, by the nitric oxide pathway. Additionally, MCT-induced PH impairs myogenic autoregulation of the diaphragm resistance vasculature. Our findings of impaired myogenic responsiveness, coupled with those of reduced endothelium-dependent and -independent vasodilation, are indicative of dysfunction induced by PH in both the endothelium and vascular

smooth muscle of diaphragm arterioles. These findings provide a potential mechanistic basis for redistribution of diaphragm perfusion in the diaphragm, predicating contractile dysfunction, dyspnea, and exercise intolerance in PH. Importantly, these findings demonstrate that a) vascular dysfunction extends beyond the pulmonary circulation and, b) that the vasculature represents at least one site of diaphragm dysfunction to therapeutically target in order to improve diaphragm function and quality of life in PH patients.

Funding: This work was supported in part by the Sustained Momentum for Investigators with Laboratories Established (SMILE) Grants awarded to TIM and DCP by Kansas State University College of Veterinary Medicine and the National Institute on Aging 1R15AG078060 awarded to BJB and DCP. AGH and KMS were financially supported by Ruth L. Kirschstein National Research Service Awards 1F31HL167618-01 and 1F31HL170643-01, respectively. ZJW and SEH were supported by Center of Biomedical Research Excellence award P20GM113109. The Johnson Cancer Research Center at Kansas State University supported supplies used in this study.

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Table 4.1 Echocardiographic measurements.

	Healthy Control		MCT-induced PH	
	Pre (n=9)	Post (n=9)	Pre (n=9)	Post (n=9)
PA Acceleration time (ms)	28 ± 5	26 ± 7	30 ± 6	19 ± 3 #
PA Ejection time (ms)	79 ± 7	78 ± 5	83 ± 3	81 ± 6
PA AT/ET	0.35 ± 0.07	0.33 ± 0.08	0.37 ± 0.07	0.24 ± 0.03 #
LV Ejection fraction (%)	83 ± 5	88 ± 6	86 ± 7	85 ± 7
LV Stroke volume (ml)	0.43 ± 0.10	0.45 ± 0.08	0.51 ± 0.10	0.52 ± 0.08
LV Fractional shortening (%)	47 ± 6	53 ± 8	51 ± 9	49 ± 7

Data are means ± SD; n, number of rats; MCT, monocrotaline; PH, pulmonary hypertension; PA, pulmonary artery; AT, acceleration time; ET, ejection time; LV, left ventricle; # P<0.05 vs. all other conditions.

Table 4.2 Animal and vessel characteristics.

	Healthy Control	MCT-induced PH
<u>Animal characteristics</u>	(n=9)	(n=9)
Body weight (g)	298 ± 29	276 ± 16
Diaphragm weight (mg)	956 ± 160	953 ± 135
Diaphragm/body weight (mg/g)	3.16 ± 0.29	3.46 ± 0.37
<u>Vessel characteristics</u>		
Spontaneous tone (%)	32 ± 12	30 ± 4
Maximal diameter (µm)	166 ± 24	178 ± 18
Maximal wall thickness (µm)	18 ± 2	18 ± 2
Wall-to-Lumen ratio (%)	11 ± 2	10 ± 2

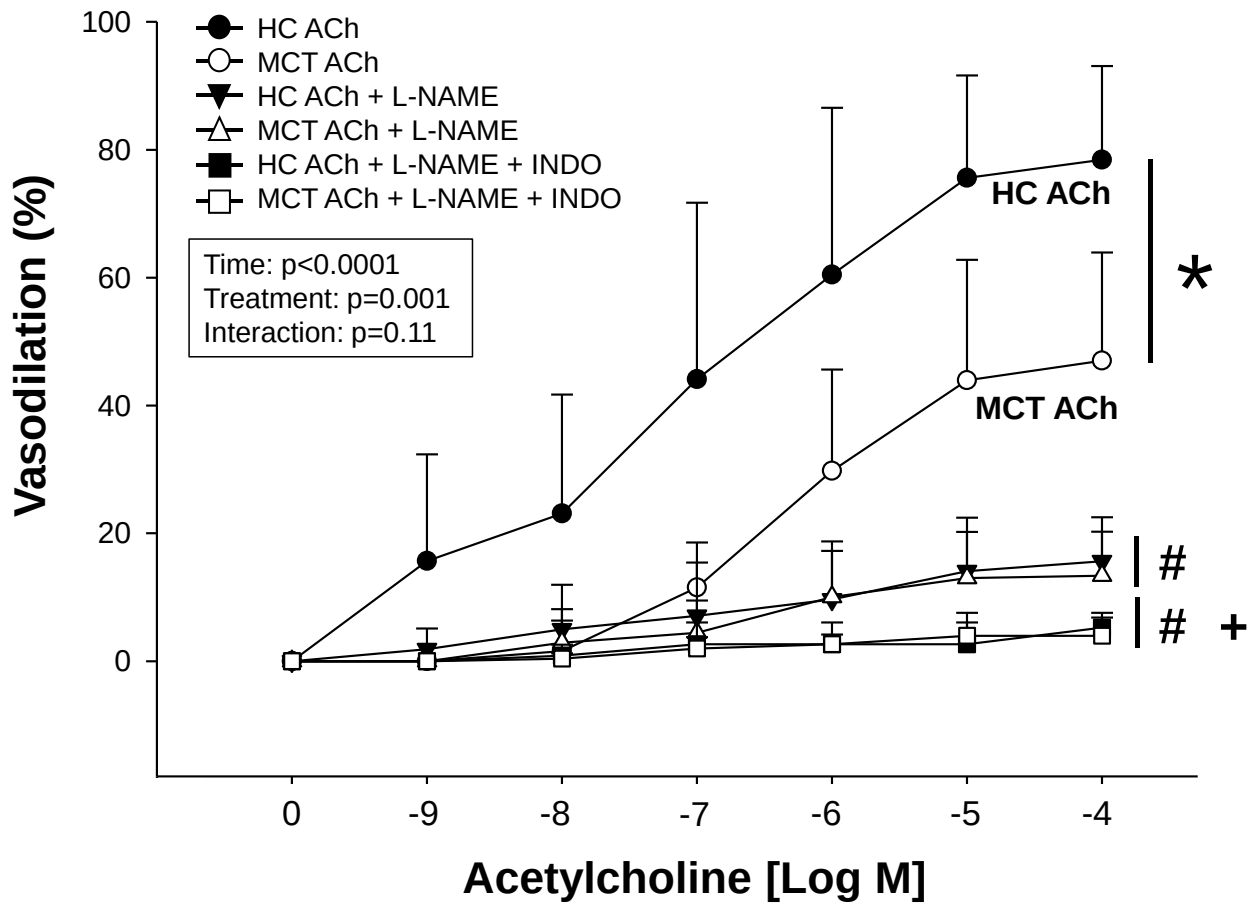
Data are means ± SD; *n*, number of rats; MCT, monocrotaline; PH, pulmonary hypertension.

Table 4.3 EC₅₀ and EC₂₅.

	Healthy Control	MCT-induced PH
<u>EC₅₀</u> (log M)		
ACh	-6.49 ± 0.88	-6.15 ± 0.94
ACh + L-NAME	-6.37 ± 1.33	-6.22 ± 0.86
ACh + L-NAME + INDO	-6.01 ± 2.06	-6.78 ± 1.14
SNP	-6.60 ± 0.86	-6.46 ± 1.01
<u>EC₂₅</u> (log M)		
ACh	-7.57 ± 0.45	-6.77 ± 0.87 #
SNP	-7.55 ± 0.69	-7.23 ± 1.21

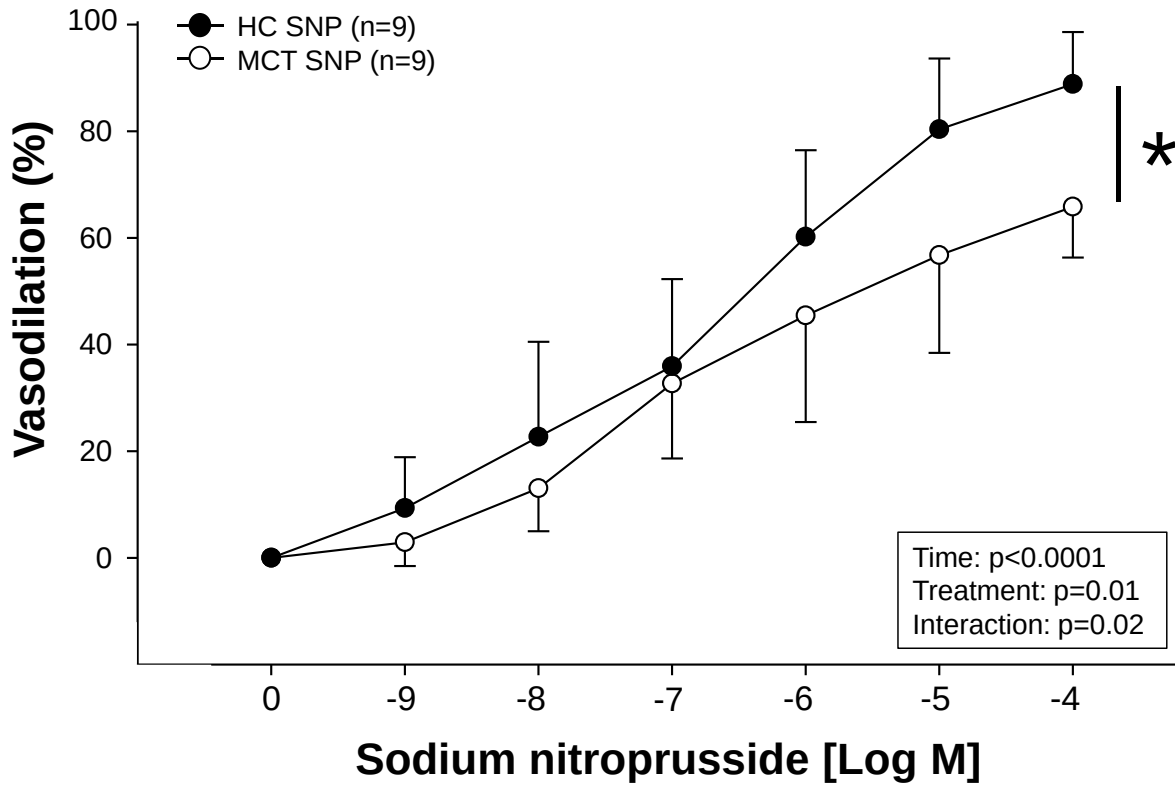
Data are means ± SD; MCT, monocrotaline; PH, pulmonary hypertension; ACh, acetylcholine; L-NAME, NG-nitro-L-arginine methyl ester; INDO, indomethacin; SNP, sodium nitroprusside; # P=0.03 vs. healthy control. Analyzed via independent two-sample t-test.

Figure 4.1 Endothelium-dependent vasodilation.



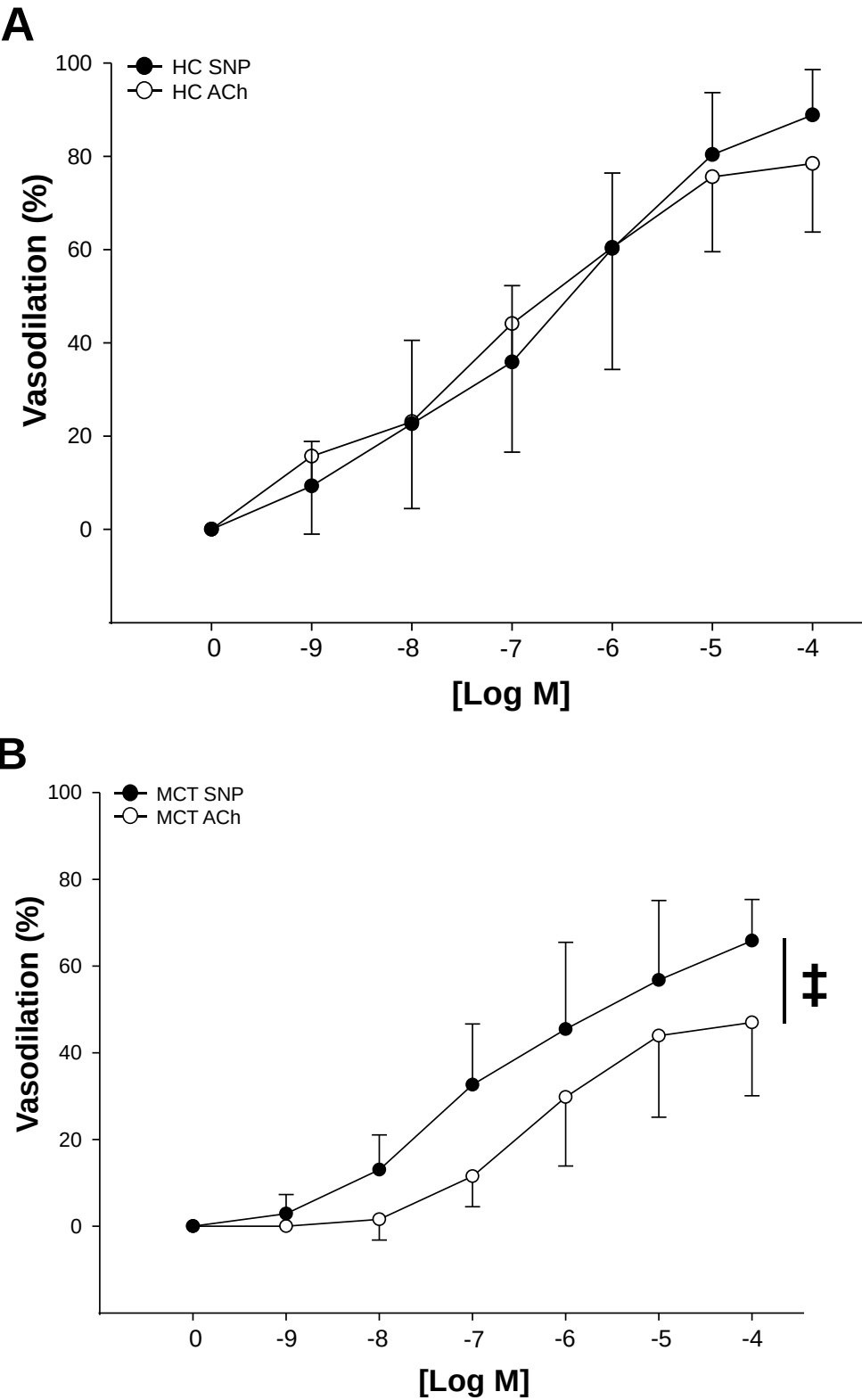
Concentration-responses to the endothelium-dependent vasodilator acetylcholine (ACh) in the absence and presence of endothelial nitric oxide synthase (eNOS) inhibitor NG-nitro-L-arginine methyl ester (L-NAME) and L-NAME + cyclooxygenase inhibitor indomethacin (INDO) in medial costal diaphragm arterioles. Sample sizes: HC ACh (n = 8), MCT ACh (n = 9), HC ACh + L-NAME (n = 7), MCT ACh + L-NAME (n = 8), HC ACh L-NAME + INDO (n = 4), MCT ACh L-NAME + INDO (n = 5). *Significant ($P < 0.05$) vs. HC ACh. #Significant ($P < 0.05$) vs. HC and MCT ACh. +Significant ($P < 0.05$) vs. HC and MCT ACh + L-NAME.

Figure 4.2 Endothelium-independent vasodilation.



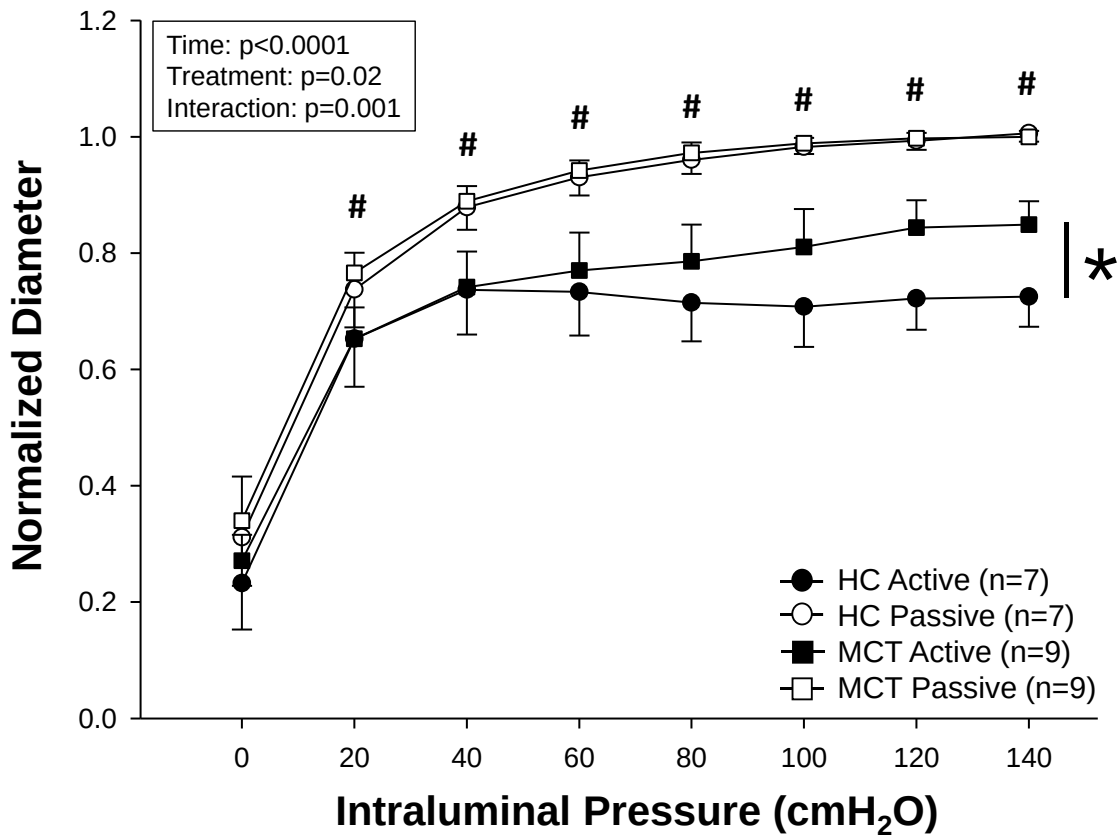
Concentration-responses to the endothelium-independent vasodilator sodium nitroprusside (SNP) in first order medial costal diaphragm arterioles. *Significant ($P < 0.05$) vs. HC SNP

Figure 4.3 Sodium nitroprusside and acetylcholine responses.



Sodium nitroprusside (SNP) and acetylcholine (ACh) concentration-responses in medial costal diaphragm arterioles from healthy control (HC) (A) and monocrotaline (MCT) induced pulmonary hypertension (B) groups. Sample sizes for A and B: HC SNP (n = 9), HC ACh (n = 8), MCT SNP (n = 9), MCT ACh (n = 9). ‡ Significant ($P < 0.05$) vs. MCT SNP.

Figure 4.4 Active and passive pressure responses.



Active and passive responses to stepwise increases in intraluminal pressure in first order medial costal diaphragm arterioles from healthy control (HC) and monocrotaline (MCT) induced pulmonary hypertension groups. *Significant ($P < 0.05$) vs. HC Active. #Significant ($P < 0.05$) vs. HC Active and MCT Active.

Chapter 5 - Skeletal and Respiratory Muscle Blood Flow

Redistribution during Submaximal Exercise in Pulmonary Hypertensive Rats

Abstract

Pulmonary hypertension (PH) is a chronic, progressive disease characterized by pulmonary vascular remodeling, dyspnea, and exercise intolerance. Key facets of dyspnea and exercise intolerance include skeletal and respiratory muscle contractile and metabolic disturbances; however, muscle perfusion during exercise has not been investigated. We hypothesized that diaphragm blood flow ($\dot{Q}$) would be increased and locomotory muscle $\dot{Q}$ would be decreased during submaximal treadmill running in PH rats compared to healthy controls. Female Sprague-Dawley rats were injected (i.p.) with monocrotaline to induce PH (n=16), or a vehicle control (n=15). Disease progression was monitored via echocardiography. When moderate disease severity was confirmed, maximal oxygen uptake ($\dot{V}O_{2\max}$) tests were performed. Rats were given >24 hours to recover, then fluorescent microspheres were infused during treadmill running (20 m/min, 10% grade) to determine tissue $\dot{Q}$. In PH rats compared with healthy controls, $\dot{V}O_{2\max}$ was lower (84(7) vs. 67(11) ml/min/kg; $P<0.001$), exercising diaphragm $\dot{Q}$ was 35% higher and soleus $\dot{Q}$ was 28% lower. Diaphragm $\dot{Q}$ was negatively correlated with soleus $\dot{Q}$ and $\dot{V}O_{2\max}$ in PH rats. Furthermore, there was regional $\dot{Q}$ redistribution in the diaphragm in PH compared to healthy, which may represent or underlie diaphragmatic weakness in PH. These findings suggest the presence of a pathological respiratory muscle blood flow steal phenomenon in PH and that this may contribute to the exercise intolerance reported in patients.

Introduction

Pulmonary hypertension (PH) is a disease characterized by pulmonary vascular remodeling, impaired gas exchange, and elevated pulmonary arterial pressures that eventually culminates into right ventricular dysfunction [Simonneau et al., 2019]. Importantly, the benefits of exercise in PH are well-recognized [Brown et al., 2017; de Man et al., 2009; Kabitz et al., 2013; Mainguy et al., 2010a; Mereles et al., 2006], but persistent dyspnea [Sun et al., 2001] and exercise intolerance [Bauer et al., 2007; Breda et al., 2014; Brown et al., 2017] often preclude regular participation in physical activity.

Exercise intolerance in PH is attributed, in part, to skeletal muscle impairments such as decreased muscle oxygenation and peripheral arterial vasoreactivity [Dimopoulos et al., 2013; Wolff et al., 2007], slowed oxygen uptake ($\dot{V}O_2$) kinetics [Barbosa et al., 2011], altered microcirculatory structure and impaired haemodynamics [Malenfant et al., 2015b; Potus et al., 2014; Schulze et al., 2022], mitochondrial dysfunction [Malenfant et al., 2015a], and an increased reliance on glycolytic metabolism [Malenfant et al., 2015a]. PH induces contractile and vascular dysfunction in the paramount respiratory muscle, the diaphragm. Specifically, PH patients exhibit impaired diaphragm strength (i.e., inspiratory and expiratory pressures and sniff velocity [Bauer et al., 2007; Breda et al., 2014; de Man et al., 2011; Meyer et al., 2006]), while rodent models demonstrate reduced diaphragm fibre contractility and in some, but not all, cases fibre atrophy [Ahn et al., 2013; de Man et al., 2011; Manders et al., 2012]. Such contractile dysfunction is likely due, in part, to compromised O_2 transport via decreased arteriolar vasoreactivity in the diaphragm of the PH rat (e.g., reduced vasodilatory capacity and myogenic constriction [Schulze et al., 2024]). These vascular changes in the diaphragm contribute to a

blunted hyperemic response to contractions and regional blood flow ($\dot{Q}$) redistribution in the anesthetized rat [Schulze et al., 2023]. Whether this redistribution persists in the conscious rat with increased ventilatory demands (e.g., exercise) has not been investigated.

Seminal recent work by Brown and colleagues [Brown et al., 2015; 2017; Long et al., 2022] demonstrated that monocrotaline (MCT)-induced PH lowers $\dot{V}O_{2\text{max}}$ and hindlimb muscle $\dot{Q}$ during submaximal exercise. However, limitations in that work leave critical knowledge and interpretational gaps: 1) skeletal muscle $\dot{Q}$ in PH and healthy rats were compared using relative exercise intensity, rather than absolute, which would be expected to lower muscle $\dot{Q}$ solely due to a lower workload, and crucially 2) diaphragm $\dot{Q}$ was not reported. During exercise in chronic left ventricular heart failure [Musch, 1993], or at high levels of respiratory muscle work [Harms et al., 1997], $\dot{Q}$ may be redirected away from working limb muscles towards the diaphragm (blood flow ‘steal’ phenomenon). Thus, examining the distribution of $\dot{Q}$ among skeletal and respiratory muscles during exercise would provide valuable information for understanding cardiovascular control during exercise in PH and whether this, at least in part, predicates exercise intolerance.

The purpose of this investigation was to measure diaphragm (bulk and regional) and skeletal muscle $\dot{Q}$ in awake PH rats at rest and during submaximal treadmill running. We hypothesized that in PH rats compared to healthy rats, there would be: 1) greater diaphragm $\dot{Q}$ at rest and during submaximal exercise, due to the chronic tachypnea and pulmonary congestion that occurs with PH, and 2) lower skeletal muscle $\dot{Q}$ during exercise and reduced $\dot{V}O_{2\text{max}}$. The results of this study will help elucidate the cardiovascular mechanisms underlying respiratory and skeletal muscle dysfunction and exercise intolerance in PH populations.

Methods

Animals and Ethical Approval

Thirty-one female Sprague-Dawley rats (4-6 months old) were obtained from Charles River Laboratories (Boston, MA, USA) for this investigation, given that the preponderance of preclinical research in PH does not reflect the sex-specific bias of the disease (4:1 female to male ratio) [Badesch et al., 2010]. Rats were randomized into healthy control (n=15) and monocrotaline (MCT)-induced pulmonary hypertension animals (PH, n=16). All procedures were approved by the Kansas State University Institutional Animal Care and Use Committee (#5019) 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 ($23 \pm 2^{\circ}\text{C}$) room with a 12:12-h light-dark cycle with water and rat chow provided ad libitum.

Monocrotaline-induced Pulmonary Hypertension

Pulmonary hypertension (PH) was induced using monocrotaline alkaloid (MCT; 50 mg/kg) administered via a single intraperitoneal injection which has been shown to induce progressive PH [Gomez-Arroyo et al., 2012; Schulze et al., 2021; 2022; 2023; 2024]. MCT (BOC Sciences; Shirley, NY) was dissolved in a solution containing 50% sterile saline and 50% 200-proof ethanol at room temperature, with 0.5 mL of this solution injected into each rat in the experimental group while healthy control rats received 0.5 mL of vehicle (50% sterile saline, 50% ethanol). While maintained on 1-2% isoflurane, injections were given immediately following pre-injection echocardiography (see Echocardiography) with a 27 G needle in the lower right quadrant of the abdomen with care taken to avoid internal organs. Body temperature was regulated at 37°C . Importantly, characteristics of PH (i.e., elevations in pulmonary artery

pressures, remodeling of small pulmonary arteries, and right ventricular (RV) hypertrophy) occur 3-4 weeks following injection, prior to the development of overt heart failure [Gomez-Arroyo et al., 2012]. Animals were monitored weekly via echocardiography along with measures of body weight and body condition scoring.

Echocardiography

Transthoracic echocardiography was performed using a commercially available system (Logiq S8; GE Health Care, Milwaukee, WI) with a 13 MHz linear transducer (L4-12t) prior to MCT or saline injection. Subsequent transthoracic echocardiography was performed weekly following injection to monitor disease progression. All comparisons were made between the pre-injection measurement and final measurement taken just prior to experimentation. Animals were initially anaesthetized by inhalation of a 5% isoflurane-O₂ mixture and maintained on $\leq 2.5\%$ isoflurane-O₂ (Butler Health Supply, Dublin, OH). Core temperature was maintained at $\sim 37^{\circ}\text{C}$, measured via rectal thermometer. B-mode and M-mode images of the left ventricle (LV) were obtained from the parasternal short axis window and analyzed for end-systolic/diastolic volumes, stroke volume, and ejection fraction as previously described [Craig et al., 2019]. RV measurements were taken at the level of the aortic valve, just proximal to the pulmonary valve, in the short axis view. Pulsed Doppler ultrasound was used to assess pulmonary artery ejection time (ET), acceleration time (AT), and peak velocity, and the ratio of AT to ET (AT/ET) was calculated and used as a clinical parameter for the confirmation of PH (AT/ET < 0.3) [Jones et al., 2002]. Upon verification of PH, the following experiments were performed.

$\dot{V}O_2\text{max}$ Testing

All rats were acclimated to running on a custom-built treadmill for ~ 5 min/day at ~ 25 m/min up a 5% grade, 5 days/week. Importantly, this acclimation protocol does not elicit training

adaptations [Armstrong & Laughlin, 1984; Musch et al., 1992]. In all treadmill running protocols, rats are encouraged to continue running via an electrical stimulator positioned at the back of the treadmill lane, as well as manual bursts of air applied beneath the hindlimbs of the animal. Maximal oxygen uptake ($\dot{V}O_{2\max}$) tests were performed in a plexiglass metabolic chamber placed on the treadmill [Musch et al., 1988]. Gas measurements were performed in real time via an O_2 (model S-3A/I) and CO_2 (model CD-3A; AEI Technologies; Pittsburg, PA) analyzer. The analyzers were calibrated before each exercise test using precision-mixed gases reflecting the expected range of gas concentrations based on previous studies [Musch et al., 1988]. Initially set at 25 m/min for 2 min, treadmill speed was increased progressively 5 m/min each minute until the rat was unable to keep pace with the treadmill or no further increases in $\dot{V}O_2$ were recorded, despite further increases in speed. Rats were weighed upon test termination, and these weights are used for relative $\dot{V}O_2$ calculations. High reproducibility of $\dot{V}O_{2\text{peak}}$ measurements have been previously recorded in our laboratory using these specific techniques [Copp et al., 2009]. In addition, $\dot{V}O_{2\text{peak}}$ has been demonstrated previously to be equal to $\dot{V}O_{2\max}$ in rats using similar exercise protocols [Bedford et al., 1979].

Haemodynamic Measurements

Surgical Preparation

All surgical procedures were performed using aseptic techniques. On the day of the final experimentation protocol, all rats were initially anesthetized with a 5% isoflurane- O_2 mixture (isoflurane vaporizer; Harvard Apparatus, Cambridge, MA) and subsequently maintained on 2.5% isoflurane- O_2 . An incision was made on the ventral side of the neck and the right jugular vein was surgically isolated and cannulated with a 2-French catheter-tip pressure micromanometer (Millar Instruments, Houston, TX, USA). The micromanometer was advanced

into the RV to obtain right ventricular systolic pressure (RVSP) via a PowerLab/LabChart data acquisition system (AD Instruments). Following RV pressure measurements, the micromanometer was withdrawn, the jugular was tied off, and the right common carotid artery was exteriorized and catheterized (PE-10 connected to PE-50, Intra-Medic polyethylene tubing; Clay Adams Brand; Becton Dickinson, Sparks, MD) for measurement of mean arterial pressure (MAP) and heart rate (HR) and infusion of fluorescent microspheres (see below). A second catheter (PE-10 connected to PE-50) was inserted into the caudal (tail) artery for arterial blood sampling. Both catheters were tunneled subcutaneously through the dorsal aspect of the cervical region and exteriorized through a puncture in the skin. The incisions were then closed, anesthesia was terminated, and the rats were given >60 min to recover [Flaim et al., 1984].

Exercising Measurements

Following recovery, each rat was placed on the motor-driven treadmill, and the carotid artery catheter was connected to the pressure transducer (PowerLab; AD Instruments). After the stabilization period, the caudal artery was connected to a 1-ml syringe chambered in an infusion/withdrawal pump (model 907; Harvard Instruments, Cambridge, MA). Treadmill speed was gradually increased to 20 m/min, 10% grade (~moderate-intensity exercise for healthy rats) and maintained for 3 minutes. During this time, fluorescent microspheres (colours: red, scarlet, or blue-green; 15.5 μm diameter; Invitrogen FluoSpheres, Carlsbad, CA) were mixed by a vortex agitator (Fisher Scientific, Waltham, MA). At the 3-min exercise mark, the carotid artery catheter was disconnected from the pressure transducer, and fluorescent microspheres ($2.0\text{-}2.5 \times 10^5$) were infused into the aortic arch. A reference blood sample was taken from the caudal artery catheter, using a Harvard withdrawal pump (model 907, Cambridge, MA) that was initiated 30 s

prior to microsphere infusion at a withdrawal rate of 0.25 ml/min. Blood withdrawal was terminated 30 s following the microsphere infusion, and then exercise was terminated.

Post-recovery Measurements

After >45 min of recovery, a second microsphere infusion was performed (fluorescence different from the first microsphere infusion) while the rat sat quietly on the treadmill for determination of resting blood flows. This experimental protocol minimizes the potential influences of the pre-exercise anticipatory response on muscle blood flows, MAP, and HR measurements [Armstrong, Hayes, & Delp, 1989]. The microspheres (red, scarlet, or blue-green) were infused in random order across animals under the two experimental conditions in which blood flows were measured [i.e., exercise and post-recovery (i.e., rest)].

Tissue Sampling and Blood Flow Determination

Following the final microsphere infusion, rats were anesthetized via inhalation (5% isoflurane-O₂ mixture) and subsequently euthanized with pentobarbital sodium overdose (>50 mg/kg I.A.) followed by cardiac excision. Thereafter, tissues (diaphragm, soleus, white portion of the gastrocnemius, intercostal muscles, and kidneys) were harvested, weighed, and placed in 15 ml screw cap polypropylene conical tubes and then placed in -80°C freezer for later blood flow analysis. The soleus and white gastrocnemius were specifically selected herein to ensure that the workload was sufficient to elicit increases in blood flow across the range of muscle fibre types as intended [Delp & Duan, 1996; Laughlin & Armstrong, 1982]. Using the template previously described [Horn et al., 2020; Horn et al., 2022; Poole et al., 1997; Schulze et al., 2023; Horn et al., 2024], the diaphragm was sectioned into costal (ventral, medial, and dorsal) and crural portions to determine regional distribution of diaphragmatic blood flow, whereas the sum of these portions was used to calculate total diaphragm blood flow.

The fluorescent microsphere assay was performed as previously described [Deveci & Eggington, 1999]. Tissue blood flow was calculated as follows:

$$\dot{Q} = [(A_t / A_b) \times (s/w)] \times 100$$

where $\dot{Q}$ is blood flow (ml/min/100g), A_t is the individual sample intensity, A_b 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). Vascular conductance was calculated as:

$$VC = \dot{Q} / MAP$$

where VC is vascular conductance (ml/mmHg/min/100g), $\dot{Q}$ is blood flow (ml/min/100g), and MAP is the average mean arterial pressure (mmHg), recorded immediately before and after microsphere infusion. Adequate mixing of microspheres was determined by <20% difference in left and right kidney or left and right soleus muscle blood flows at each time point. Where inadequate mixing sample was present in any animal or condition, that data was removed. Other reasons for animal exclusion include technical challenges during the blood flow assay, improper catheter placement, or no withdrawal from either carotid or tail artery catheters.

Postmortem Measurements

The RV, LV, and lungs were dissected and weighed following euthanasia. RV hypertrophy was measured via the Fulton index: RV weight (mg) / LV + septum (S) weight (mg) [Fulton et al., 1952]. RV hypertrophy was also expressed as RV/body weight and RV/tibial bone length.

Statistical Analysis

Data were analyzed using GraphPad Prism9 (GraphPad Software, San Diego, CA). Student paired (within animal) and independent two-sample (between healthy and PH) t-tests were utilized to determine differences in $\dot{V}O_{2max}$, RVSP, and morphometric measurements.

Echocardiographic measurements were compared using a two-way ANOVA. Tissue blood flows were analyzed with a mixed-effects model. A Grubb's outlier test was performed on all data, and 1 outlier was identified and removed from the respective data subset when detected prior to statistical analysis. Post-hoc analyses were performed using a Tukey's test. All data are presented as means (SD). Significance was established at $P < 0.05$.

Results

Morphometric and Echocardiographic Data

PH rats displayed increased lung weight, lung/body mass, lung/tibial bone length, RVSP, RV/LV+S, RV/body mass, and RV/tibial bone length compared to healthy rats ($P < 0.05$; Table 5.1). There were no differences in RV or LV echo parameters (Table 5.2) or body mass (288(15) vs. 282(13) g) prior to injection (all $P > 0.05$). At the time of final measurements, body mass, PA AT, and AT/ET were reduced in PH rats ($P < 0.05$; Tables 5.1 & 5.2), with no differences in LV echo measurements (all $P > 0.10$; Table 5.2).

$\dot{V}O_{2max}$

PH rats had a lower $\dot{V}O_{2max}$ compared to healthy counterparts (Figure 5.1). There were also significant differences in $\dot{V}CO_{2max}$ (85(7) vs. 72(13) ml/kg/min; $P = 0.009$), final running speed (49(6) vs. 43(6) m/min; $P = 0.004$), and total running time (405(88) vs. 315(89) seconds; $P = 0.009$) between healthy and PH rats, respectively. There were no differences in pre-exercise $\dot{V}O_2$ (27(4) vs. 24(7) ml/kg/min; $P = 0.165$), $\dot{V}CO_2$ (27(5) vs. 27(6) ml/kg/min; $P = 0.786$), or RER at $\dot{V}O_{2max}$ (1.03(0.08) vs. 1.09(0.10); $P = 0.137$).

Resting and Exercising Blood Flow

At rest, there were no differences between groups in bulk diaphragm $\dot{Q}$ or vascular conductance (VC), or skeletal muscle $\dot{Q}$ or VC (Figures 5.2 & 5.3). During submaximal

exercise, skeletal muscle (i.e., soleus and white portion of gastrocnemius) $\dot{Q}$ and VC increased in healthy rats; however, in PH rats, only soleus muscle blood flow increased from rest to exercise, with PH rats displaying lower exercising $\dot{Q}$ in both muscles (Soleus: $P = 0.025$, WG: $P = 0.073$) compared with healthy animals (Figure 5.2). Diaphragm $\dot{Q}$ and VC increased from rest to exercise in both groups. Diaphragm $\dot{Q}$ and VC were higher in PH vs. healthy rats during exercise (Figure 5.3). In both groups, $\dot{Q}$ increased in all regions from rest to exercise ($P < 0.05$) and was highest in the medial costal portion of the diaphragm, followed by ventral, dorsal, and then crural. Differences between healthy and PH were present in the medial costal and crural regions at rest, and the ventral costal, medial costal, and crural regions during exercise (Figure 5.4). There were no differences in intercostal $\dot{Q}$ (21(17) vs. 27(13) ml/min/100g; $P = 0.446$) or VC (0.16(0.13) vs. 0.23(0.11) ml/mmHg/min/100g; $P = 0.330$) at rest between healthy and PH, respectively. $\dot{Q}$ and VC to intercostal muscles increased from rest to exercise only in PH rats ($\dot{Q}$: 47(28) ml/min/100g, VC: 0.38(0.22) ml/mmHg/min/100g; $P = 0.043$ vs. rest for both), but not healthy controls ($\dot{Q}$: 37(29) ml/min/100g, VC: 0.29(0.21) ml/mmHg/min/100g; $P = 0.104$ vs. rest for both). HR increased with exercise in both groups, with no differences between groups at rest (395(44) vs. 392(60) bpm) or during exercise (475(59) vs. 459(67) bpm). MAP was higher in healthy vs. PH rats at rest (132(12) vs. 118(16); $P < 0.001$) and during exercise (131(8) vs. 116(17); $P < 0.001$). No differences were detected in kidney $\dot{Q}$ between groups or conditions with values ranging from 500-800 ml/min/100g, as previously reported [Ferguson et al., 2016; Holdsworth et al., 2015; Musch & Terrell, 1992]. In PH rats, diaphragm $\dot{Q}$ during submaximal exercise was negatively correlated with both $\dot{V}O_{2\max}$ and soleus muscle $\dot{Q}$ during exercise (Figures 5.5A and 5.5B), and $\dot{V}O_{2\max}$ was negatively correlated with submaximal exercising blood [lactate] (Figure 5.5C).

Discussion

The key original findings of this investigation are that skeletal muscle $\dot{Q}$ during exercise is lower while diaphragm $\dot{Q}$ is higher in the MCT model of PH compared with healthy controls, suggesting the presence of a pathological $\dot{Q}$ ‘steal’ phenomenon. Further, $\dot{V}O_{2\max}$ is reduced in PH, relative to the extent to which diaphragm $\dot{Q}$ is raised during submaximal exercise. These results support a relationship between diaphragmatic recruitment and exercise intolerance and demonstrate the adaptive $\dot{Q}$ responses which allows elevated ventilatory work to be sustained during exercise in PH preceding right ventricular failure.

The monocrotaline model of PH has been demonstrated in our lab [Schulze et al., 2021; 2022; 2023; 2024] and others [Brown et al., 2015; Brown et al., 2017; de Man et al., 2011; Gomez-Arroyo et al., 2012; Long et al., 2022] to induce similar cardiopulmonary characteristics found in human pathology. Specifically, PH animals herein presented with reduced AT/ET, elevated RVSP, and increased lung weights indicative of pulmonary congestion (Tables 5.1 and 5.2). The $\dot{V}O_{2\max}$ values in this investigation for healthy animals correspond closely to those found previously in healthy rats using these protocols established in our laboratory (~75-80 ml/kg/min; Copp et al., 2009). In addition, soleus, diaphragm, intercostal, and kidney $\dot{Q}$ at rest and during exercise in health and PH are broadly consistent with those previously reported in healthy and chronic heart failure rats, respectively [Copp et al., 2010; Holdsworth et al., 2015; Musch & Terrell, 1992; Musch, 1993].

Blood Flow and Vascular Conductance

Cardiac output is a prognostic indicator of survival in PH [Sandoval et al., 1994], likely related to its inextricable link with exercise capacity. In moderate PH preceding right ventricular failure, it is less likely that there will be reduced exercising cardiac output than that which may

be present in a more severe disease phenotype; however, an altered distribution of cardiac output would significantly impact tissue perfusion and spatial O₂ matching, which would decrease exercise capacity prior to reductions in cardiac output. The measurement of skeletal and respiratory muscle $\dot{Q}$ in the current study permits greater understanding of the distribution of cardiac output among and within active muscle during exercise in PH.

In the present investigation, bulk diaphragm $\dot{Q}$ and vascular conductance were higher in PH rats during treadmill running compared to healthy rats, while locomotory muscle $\dot{Q}$ was lower. This supports our hypothesis and agrees with previous findings in chronic left ventricular heart failure [Musch & Terrell, 1992; Musch, 1993]. Our results regarding lower skeletal muscle $\dot{Q}$ during submaximal exercise in moderate PH corroborate those of Long et al. [2022] in rats with severe PH at relative exercise intensities. Importantly, utilizing relative workloads invariably produces lower exercising metabolic rates and hindlimb muscle $\dot{Q}$ in PH due to lower running speed; therefore, it cannot be determined whether the lower hindlimb $\dot{Q}$ found by Long et al. is a result of PH or simply a lower workload. The same absolute workload was selected herein because PH patients and healthy individuals perform daily tasks that are equivalent in absolute terms, thus representing a higher percentage of their absolute $\dot{V}O_{2\max}$. In the current investigation, rats ran at 40% (Healthy) and 48% (PH) of their maximal speed attained during their $\dot{V}O_{2\max}$ test ($P = 0.007$). Despite running at a higher relative workload, PH rats received less blood flow to working locomotory muscles, which we surmise may be due to the heightened diaphragm $\dot{Q}$ (Figure 5.5C). As demonstrated by Harms and colleagues, experimentally increasing the work of breathing contributes to respiratory muscle $\dot{Q}$ ‘steal’ from locomotory muscles, lowering exercise capacity, even in healthy, trained subjects [Harms et al., 1997; 2000]. Herein, exacerbated lactate accumulation, higher arterial PaCO₂, and lower PaO₂ during exercise

(Table 5.3) would be expected to increase ventilatory demand above that of healthy animals via greater carotid body stimulation. Indeed, the augmented diaphragm $\dot{Q}$ would suggest that the work of breathing, largely powered by the diaphragm, is higher in PH rats during exercise. Previous investigations have identified relationships between exercise intolerance, ventilatory insufficiency [Sun et al., 2001], and respiratory muscle dysfunction in PH patients [Bauer et al., 2007; Breda et al., 2014]. Our findings suggest that the degree to which diaphragm $\dot{Q}$ must be elevated to maintain an increased work of breathing may serve as at least one mechanism underlying these relationships (Figure 5.5). We postulate that the elevated diaphragmatic energetic demands imposed by PH result in pathological respiratory muscle $\dot{Q}$ steal, even at the submaximal workloads demonstrated herein, and may contribute to the exercise intolerance in PH; however, the neural, humoral, and vascular control mechanisms that coordinate such $\dot{Q}$ redistribution away from locomotory muscle in PH require further investigation.

While there was no effect of PH on bulk diaphragm $\dot{Q}$ at rest, assessment of regional $\dot{Q}$ distribution revealed a group difference (Healthy vs. PH) at rest and during exercise (Figure 5.4), suggesting that differences in resting diaphragm $\dot{Q}$ were masked by simply analyzing bulk $\dot{Q}$. During exercise, higher diaphragm $\dot{Q}$ in PH was driven by the elevated ventral costal, medial costal, and crural $\dot{Q}$ compared to healthy rats (Figure 5.4), markedly analogous to emphysematous hamsters [Sexton & Poole, 1998]. Although higher diaphragm $\dot{Q}$ in PH was expected, the mechanisms by which bulk diaphragm $\dot{Q}$ was increased by a similar magnitude as in healthy rats (~130% from rest) are somewhat unclear, given the medial costal vascular dysfunction and impaired hyperemia that has been previously demonstrated in PH rats [Schulze et al., 2023; 2024]. The hyperemic response within each region may be the key to understanding this phenomenon. In healthy rats, the ventral costal and crural regions had the lowest hyperemic

response, indicating marginal recruitment from rest to submaximal exercise, whereas the medial costal has the most robust response, followed by dorsal costal, as expected [Sexton & Poole, 1995] (Figure 5.4E). But in PH, despite a larger total increase in blood flow, the hyperemic response of the medial costal and dorsal costal (as a proportional increase from rest) is less compared to healthy animals, with a greater increase in ventral costal $\dot{Q}$ (Figure 5.4E). These results are consistent with the notion that, in the presence of medial costal vascular dysfunction, other regions (Figures 5.4B and 5.4D) may be recruited more heavily in PH to maintain the total diaphragm $\dot{Q}$ necessary to sustain ventilatory work [Schulze et al., 2023; 2024]. Although total respiratory muscle $\dot{Q}$ does not appear to be a limitation to submaximal exercise herein, it is unknown whether there is a ‘ceiling effect’ that may become apparent at maximal exercise intensities. Interestingly, $\dot{Q}$ and vascular conductance to the intercostal muscles were increased from rest to exercise in PH rats only, akin to both emphysematous hamsters and aged rats [Smith et al., 2019; Sexton & Poole, 1998], suggesting a greater reliance on accessory respiratory muscles during exercise in PH compared to healthy rats.

Exercise Intolerance in PH

Our findings confirm those of others that PH reduces exercise tolerance in animal models [Long et al., 2022, MCT; Zhang et al., 2024, Sugan-Hypoxia], as it does in PH patients [Mainguy et al., 2010; Malenfant et al., 2015b]. Herein, $\dot{V}O_{2\max}$ values in PH rats were 50-60 ml/kg/min (~75% of healthy) and are consistent with prior investigations supporting that lower exercise capacity in PH may be due to alterations or impairments in the O_2 transport pathway. Specifically, these include compromised skeletal muscle mitochondrial structure/function [Malenfant et al., 2015a], capillarity [Malenfant et al., 2015b; Potus et al., 2014], oxygenation [Dimopoulos et al., 2013; Schulze et al., 2021], intrinsic muscle function or fibre type [in some-

Batt et al., 2014; Breda et al., 2014; Mainguy et al., 2010b; but not all- Zhang et al., 2024], $\dot{V}O_2$ kinetics [Barbosa et al., 2011], and vascular function [Long et al., 2022; Wolff et al., 2007], as well as central cardiopulmonary factors.

Elevated blood [lactate] herein supports that both groups were performing in the heavy intensity domain. Nonetheless, blood [lactate] was increased to a greater extent in PH compared to healthy controls (Table 5.3), which was negatively correlated with $\dot{V}O_{2\max}$ in PH rats (Figure 5.5C). The exaggerated lactate accumulation may be attributed to lower skeletal muscle $\dot{Q}$, the higher relative workload, the expected sympathetic stimulation and catecholamine response, greater reliance upon glycolytic metabolism, and/or increased lactate dehydrogenase activity in skeletal muscle [Malenfant et al., 2015a]. As such, this would inevitably contribute to early-onset fatigue and serve to limit exercise tolerance in moderate-severity PH.

As developed by Wagner [1996] and Roca et al. [1992], $\dot{V}O_{2\max}$ is determined by perfusive ($\dot{Q}O_2$) and diffusive (DO_2) components of oxygen delivery. The results of our study suggest that skeletal muscle $\dot{Q}$ is impaired during submaximal exercise in PH. We have previously demonstrated impairments in capillary haemodynamics in skeletal muscle from PH rats at rest, which support that both perfusive and diffusive components may play a role in lowering $\dot{V}O_{2\max}$ in PH [Schulze et al., 2022]. Crucially, low O_2 delivery may push PH into the O_2 -dependent zone [Poole et al., 2012 for Rev.], slowing $\dot{V}O_2$ kinetics [Barbosa et al., 2011] and predicating exercise intolerance. These phenomena occurring in PH patients may preclude their participation in daily physical activities or exercise training [Mainguy et al., 2011], which is unfortunate considering that these therapies have proven efficacy in mitigating PH symptoms and pathogenesis [Brown et al., 2015; Brown et al., 2017; de Man et al., 2009; Kabitz et al., 2013; Mainguy et al., 2010a; Mereles et al., 2006].

Experimental Considerations

This investigation, by design, utilized a mild to moderate-severity model of MCT-induced PH to help resolve the effects of PH independent of RV failure. MCT is metabolized in the liver and subsequently induces pulmonary vascular remodeling, imitating the PH pathophysiology. The effects of MCT independently on peripheral vascular function or the diaphragm itself have not been directly investigated and should be considered when interpreting these findings. Future investigations may seek to compare different models of PH in this regard. As with all animal models, translation to human pathology should be employed with caution.

Conclusions

Our findings support the notion that skeletal muscle blood flow during submaximal exercise is reduced in PH, and critically, that diaphragm (and intercostal) blood flow is increased, representing a pathological ‘respiratory muscle steal’ phenomenon in PH, compromising exercise capacity. Furthermore, with elevated ventilatory demand, blood flow is redistributed towards potentially less mechanically efficient regions of the diaphragm (ventral costal and crural) in PH, which may underlie diaphragmatic weakness and failure in PH. These data illustrate that the system will prioritize meeting ventilatory needs at the expense of locomotory muscle function. Notably, this occurs in moderate-severity PH suggesting that these effects manifest prior to the development of right ventricular failure. The specific neural, humoral, or vascular mechanisms which coordinate such blood flow redistribution among skeletal and respiratory muscle in PH require further investigation.

Funding: This work was supported in part by the Sustained Momentum for Investigators with Laboratories Established (SMILE) Grants awarded to T.I.M and D.C.P. by Kansas State

University College of Veterinary Medicine and the National Institute on Aging 1R15AG078060 awarded to BJB and DCP. AGH and KMS were financially supported by NIH NHLBI Ruth L. Kirschstein National Research Service Awards 1F31HL167618-02 and 1F31HL170643-02, respectively. The Johnson Cancer Research Center at Kansas State University supported supplies used in this study

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Table 5.1 Morphometric and Millar measurements.

	Healthy Control	MCT-induced PH
<u>Animal characteristics</u>	(n=15)	(n=16)
Body weight (g)	316 (19)	287 (20) *
Diaphragm (mg)	930 (82)	877 (86)
Diaphragm/body weight (mg/g)	3.0 (0.3)	3.1 (0.3)
RV (mg)	176 (17)	187 (32)
LV+S (mg)	709 (58)	638 (71) *
RV/LV+S	0.25 (0.02)	0.29 (0.05) *
RV/body weight	0.56 (0.06)	0.66 (0.13) *
Lungs (mg)	1149 (112)	1905 (870) *
Lung/body weight (mg/g)	3.66 (0.47)	6.77 (3.4) *
RV/tibial bone length (mg/mm)	4.1 (0.4)	4.5 (0.8) *
Lung/tibial bone length (mg/mm)	27.0 (3)	48.1 (21) *
RVSP (mmHg)	22 (2)	31 (11) *

Data are means (SD); *n*, number of rats; MCT, monocrotaline; PH, pulmonary hypertension; *

P<0.05 vs. healthy control

Table 5.2 Echocardiographic measurements.

	Healthy Control		MCT-induced PH	
	Pre (n=15)	Post (n=15)	Pre (n=16)	Post (n=16)
PA Ejection time (ms)	81 (3)	78 (5)	82 (6)	81 (8)
PA Acceleration time (ms)	27 (5)	29 (6)	26 (3)	20 (3) [#]
PA AT/ET	0.33 (0.06)	0.36 (0.06) *	0.32 (0.04)	0.24 (0.03) [#]
LV Ejection fraction (%)	80 (7)	83 (5)	84 (8)	84 (6)
LV Stroke volume (ml)	0.55 (0.12)	0.58 (0.11)	0.59 (0.11)	0.53 (0.11)
LV Fractional shortening (%)	44 (7)	47 (6)	49 (9)	48 (7)

Data are means (SD); n, number of rats; MCT, monocrotaline; PH, pulmonary hypertension; PA, pulmonary artery; AT, acceleration time; ET, ejection time; LV, left ventricle; * P = 0.041 vs.

Pre within group; # P<0.001 vs. all other conditions

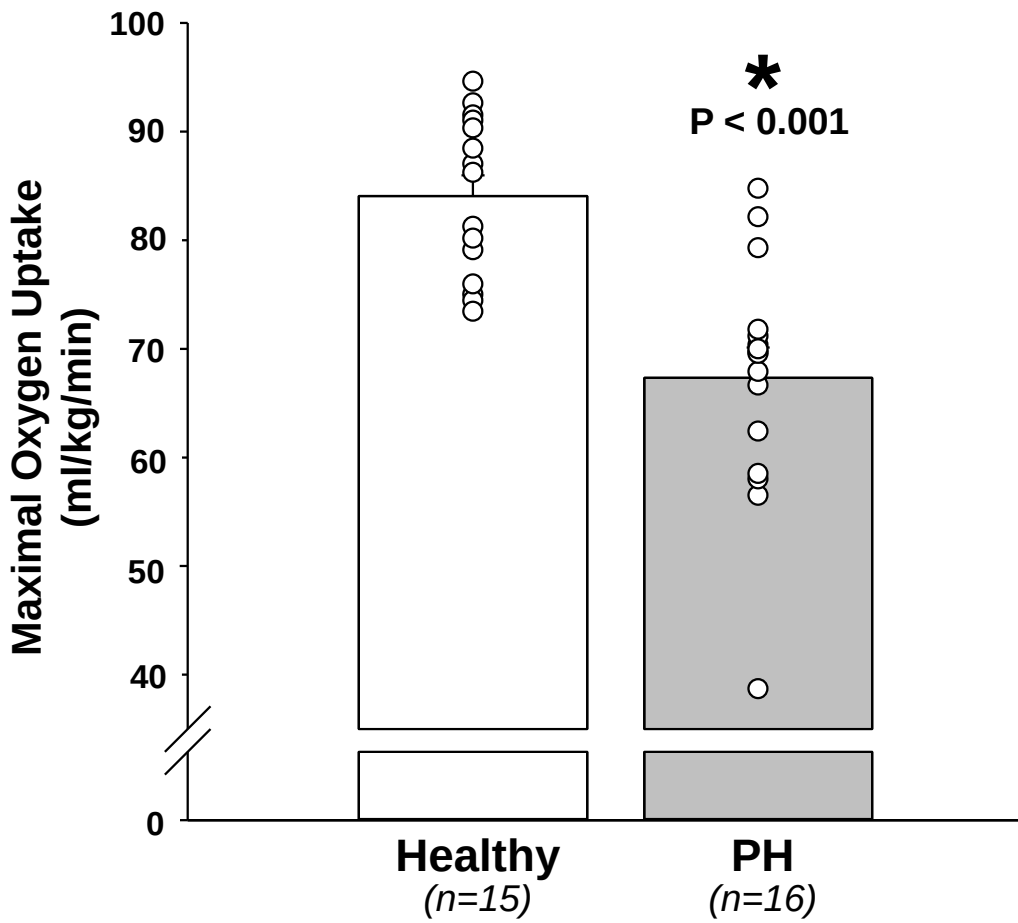
Table 5.3 Arterial blood gas data.

	Healthy Control	MCT-induced PH
<i>Rest</i>	(n=14)	(n=13)
pH	7.45 (0.04)	7.45 (0.04)
PCO ₂ (mmHg)	23.2 (3.3)	25.9 (4.6)
PO ₂ (mmHg)	112.7 (10.0)	92.8 (29.6) *
SaO ₂ (%)	99.6 (0.5)	93.5 (9.5)
Hct (%)	27.6 (4.4)	27.8 (5.9)
Lactate (mmol/L)	0.55 (0.21)	0.66 (0.19)
<i>Exercise</i>		
pH	7.44 (0.03)	7.39 (0.12) #
PCO ₂ (mmHg)	19.8 (2.9)	26.0 (10.3) *
PO ₂ (mmHg)	119.5 (11.9)	92.3 (31.9) *
SaO ₂ (%)	99.6 (0.5)	87.3 (21.8) *
Hct (%)	30.4 (4.6)	32.8 (4.8) #
Lactate (mmol/L)	2.8 (0.7) #	5.5 (3.3) * #

Data are means (SD); *n*, number of rats; MCT, monocrotaline; PH, pulmonary hypertension; *

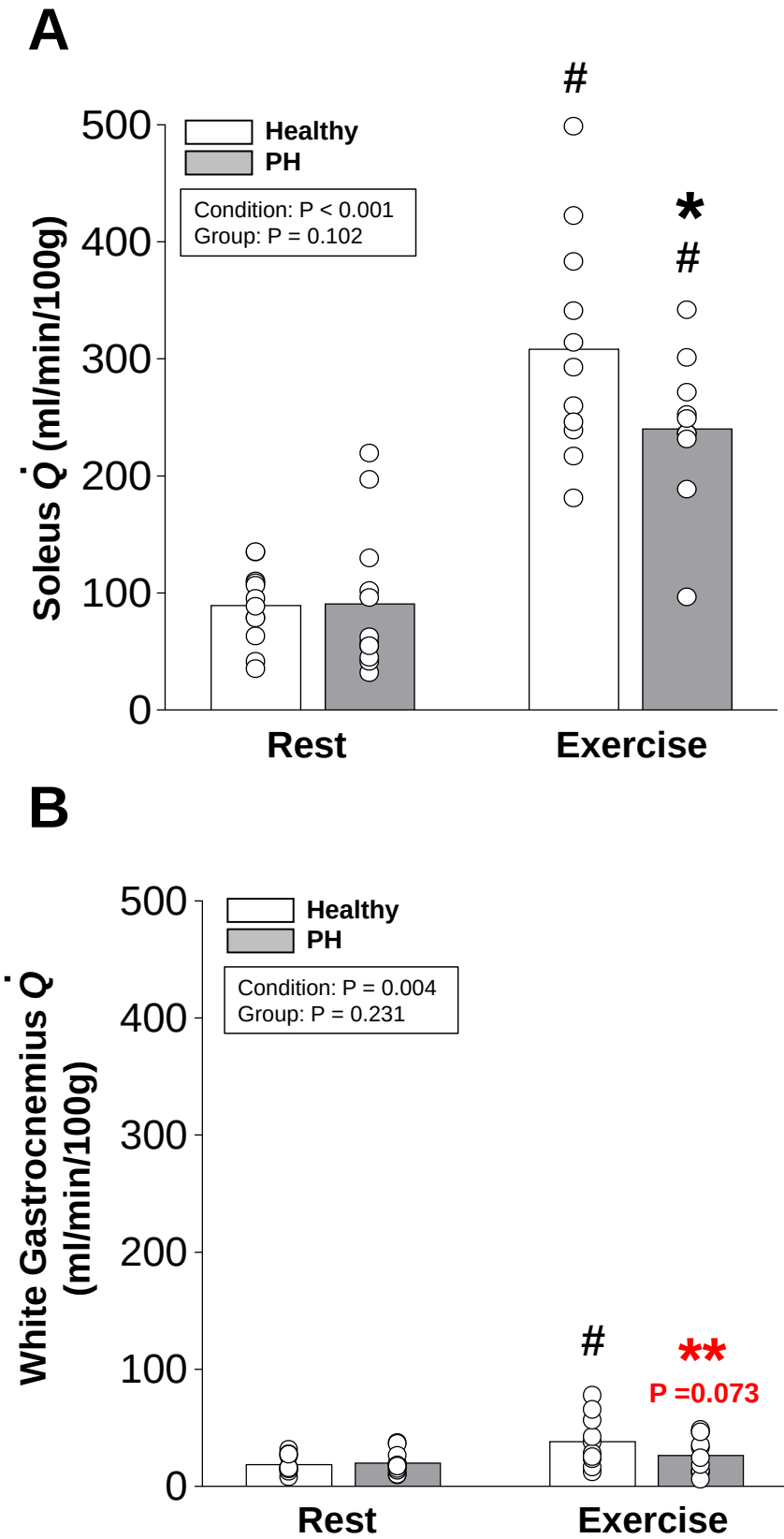
P<0.05 vs. healthy control, # P<0.05 vs. rest within group

Figure 5.1 Maximal oxygen uptake.



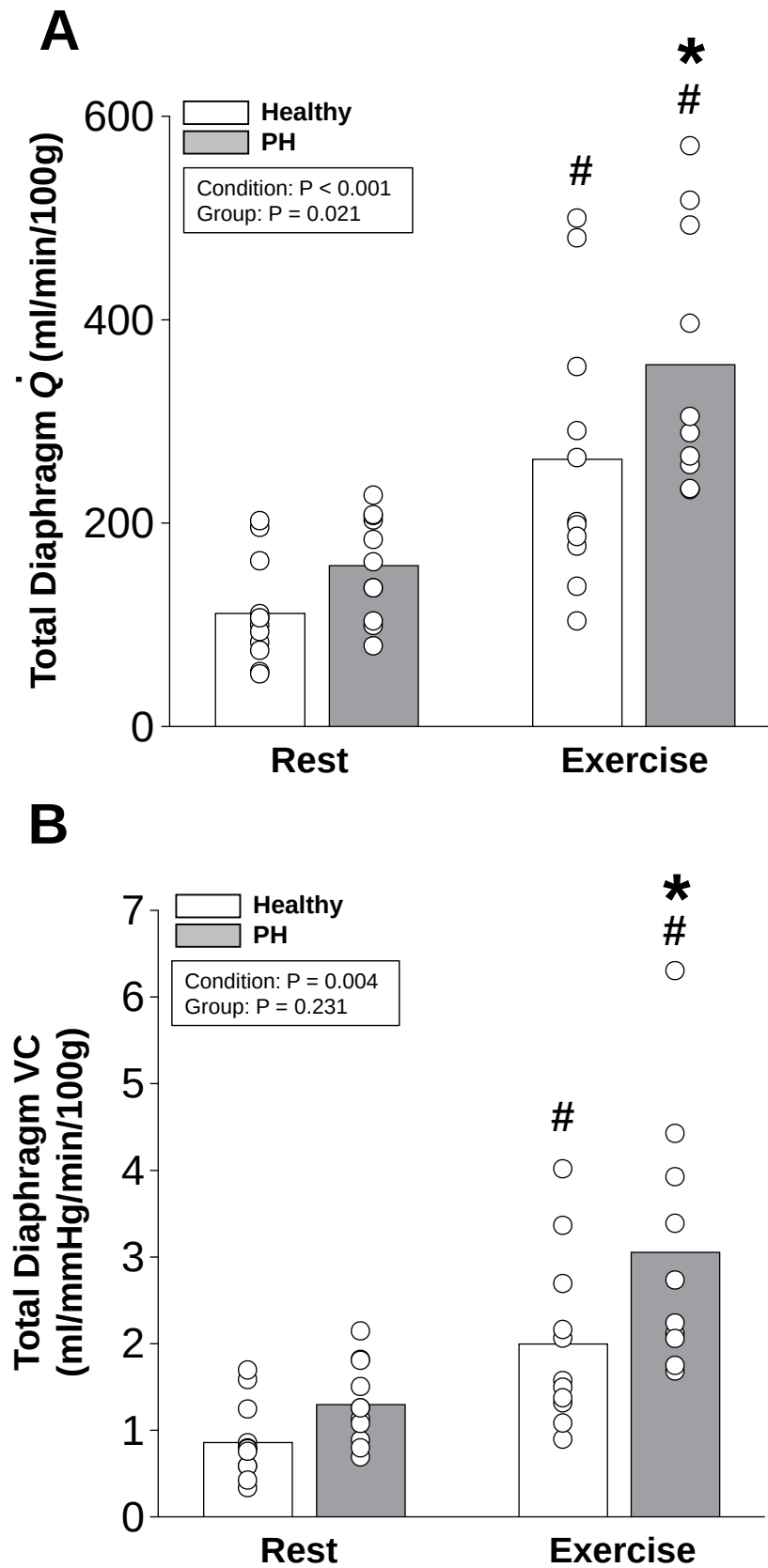
$\dot{V}O_{2\text{max}}$ in healthy and PH rats.

Figure 5.2 Locomotory muscle blood flow.



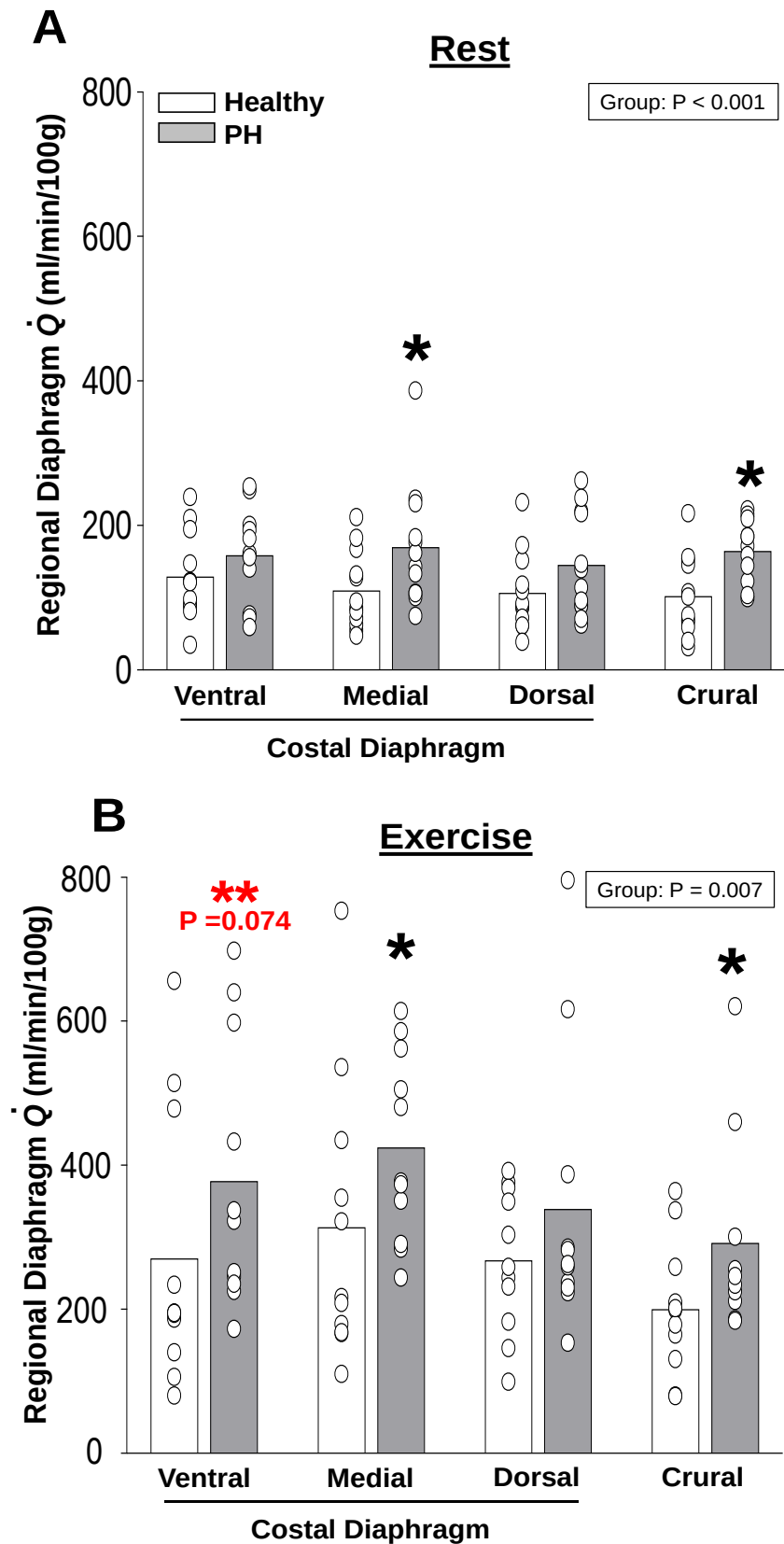
Hindlimb muscle blood flow. Soleus (A) and WG blood flow ($\dot{Q}$) (B) at rest and during exercise in healthy (white bar) and PH (grey bar). # $P < 0.05$ vs. rest within group. * $P = 0.025$ vs. healthy within condition. ** $P = 0.073$ vs. healthy within condition. In both groups, $n=12$ at rest and $n=11$ during exercise.

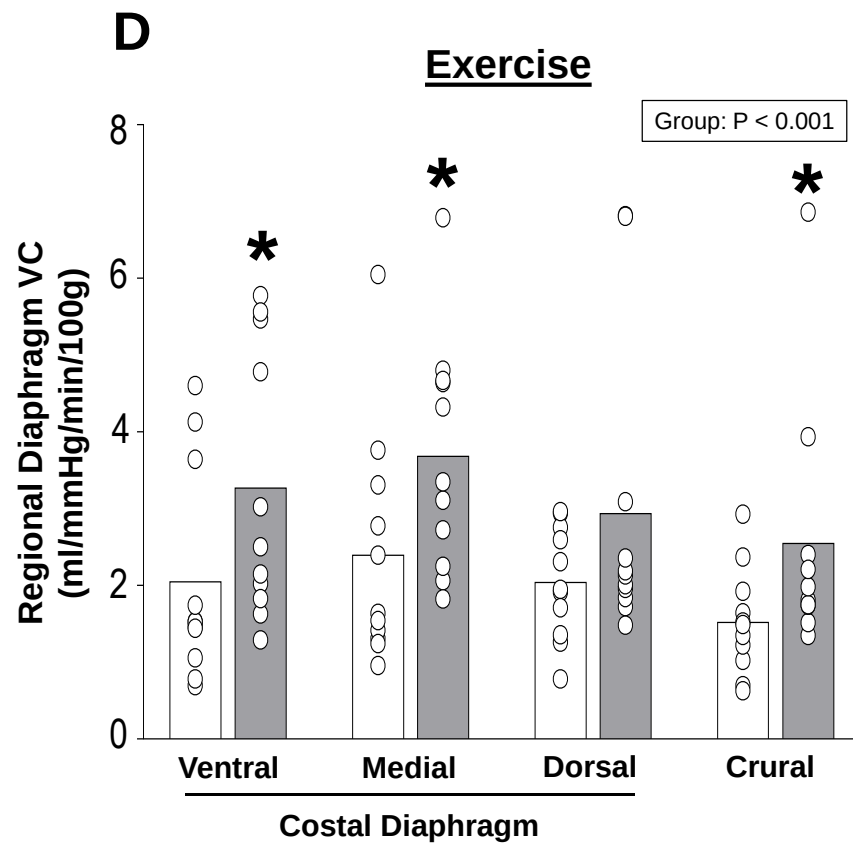
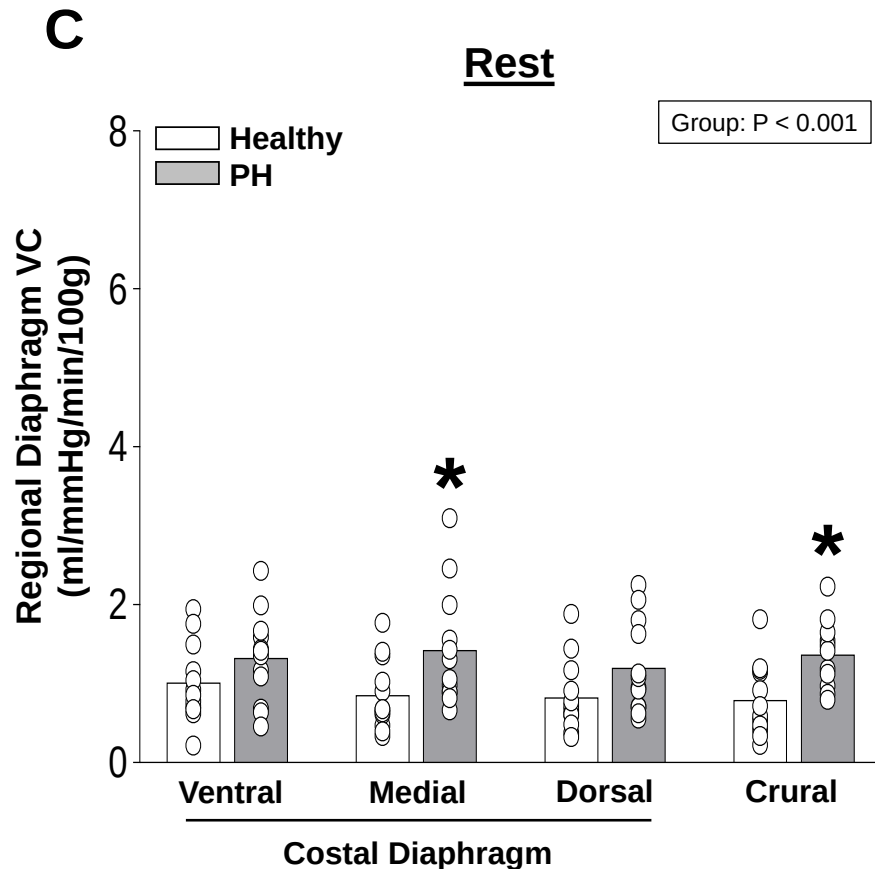
Figure 5.3 Bulk diaphragm blood flow and vascular conductance.



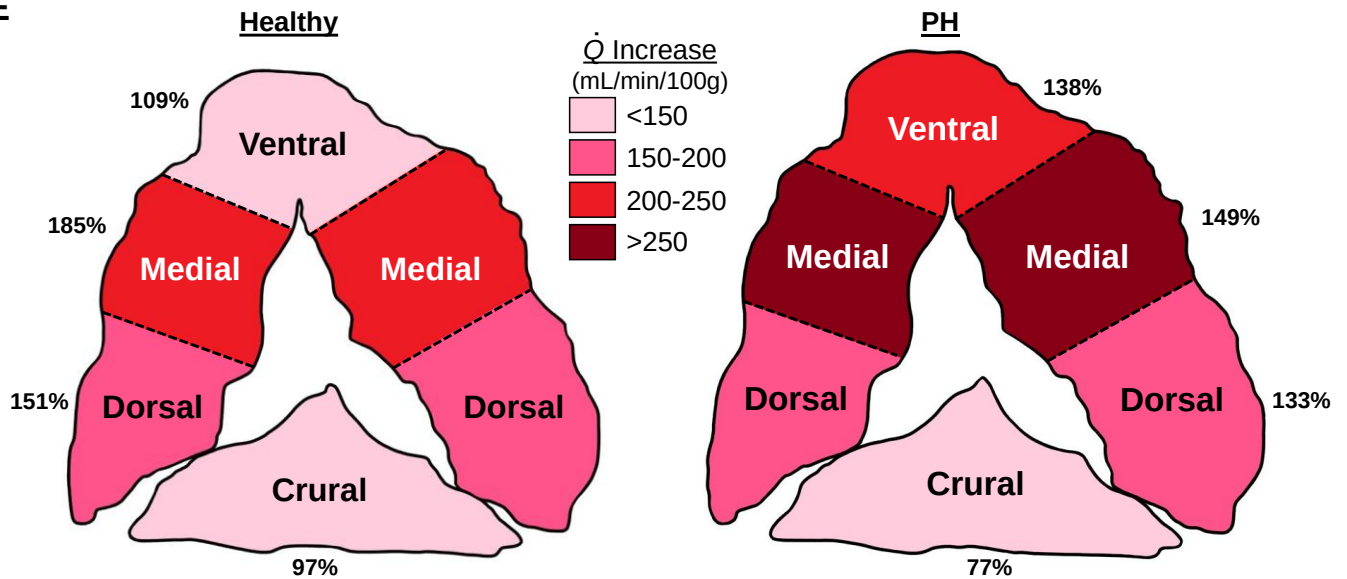
Total diaphragm blood flow ($\dot{Q}$) (A) and vascular conductance (VC) (B) in healthy (white) and PH (grey) bars. # $P < 0.05$ vs. rest within group. * $P < 0.05$ vs. healthy within condition. In both groups, $n=12$ at rest and $n=11$ during exercise.

Figure 5.4 Regional diaphragm blood flow and vascular conductance.



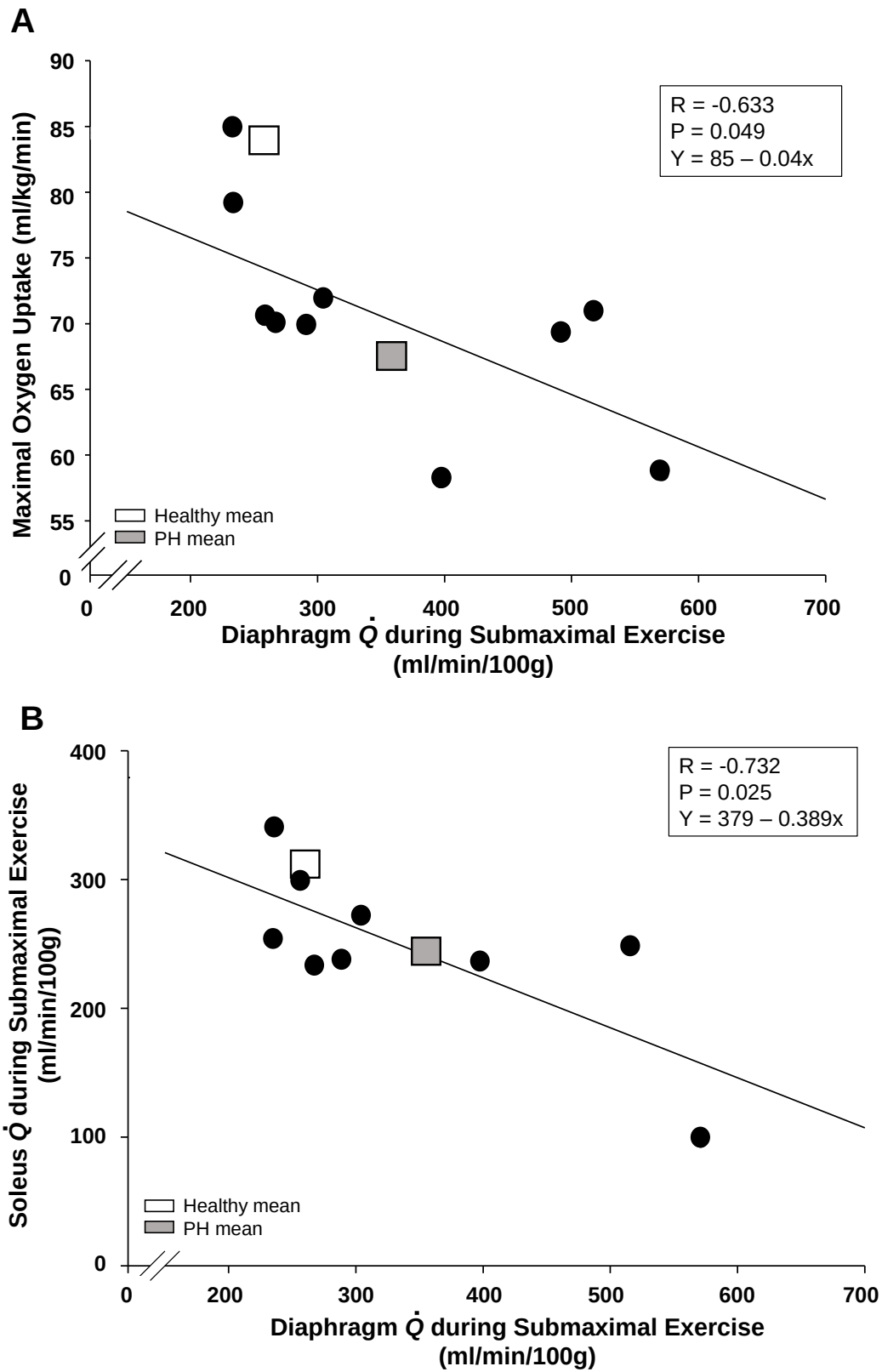


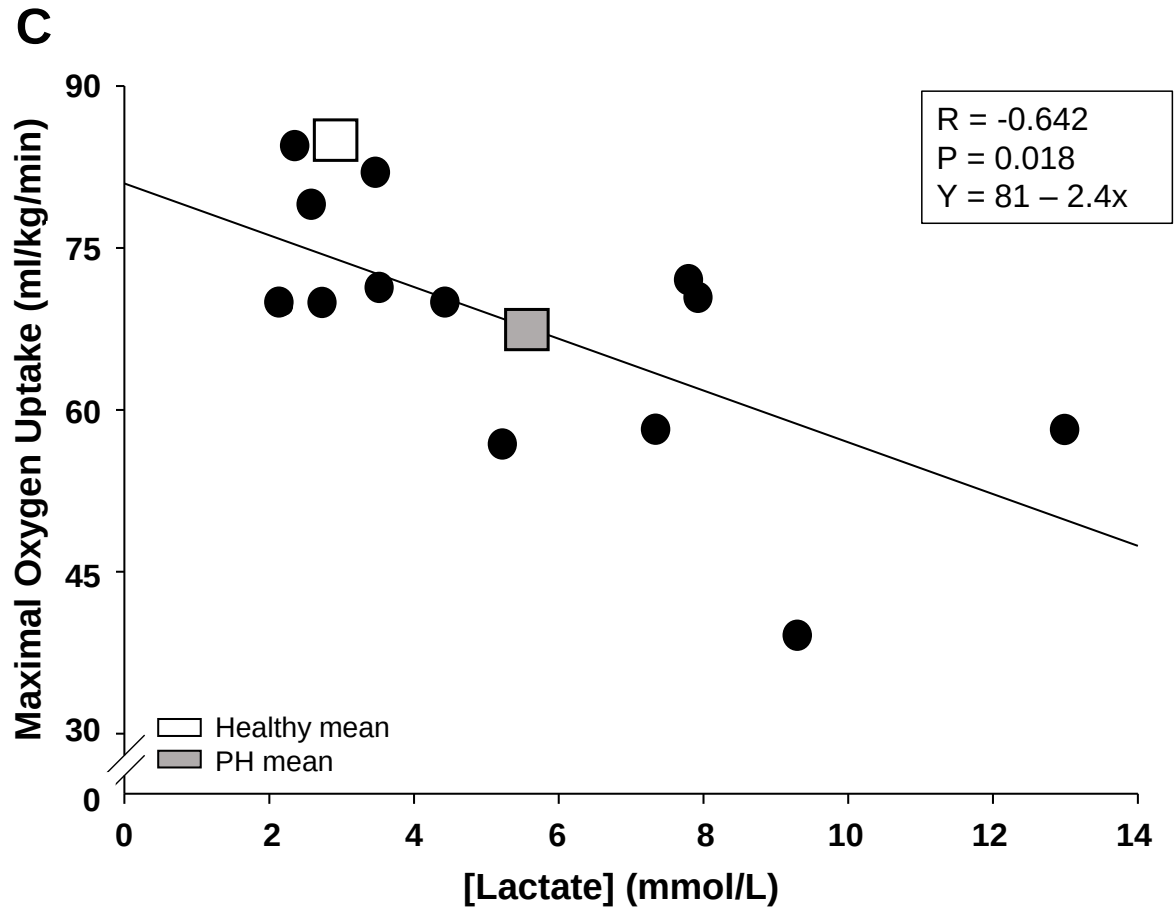
E



Regional diaphragm blood flow ($\dot{Q}$) and vascular conductance (VC) at rest (A & C) and during submaximal treadmill exercise (B & D) in healthy (white bar) and PH (grey bar). * $P < 0.05$ vs. healthy within region. ** $P=0.074$ vs. healthy within region. (E) Visual representation of regional exercise-induced hyperemia in healthy and PH rats (Absolute blood flow increase from resting value; percent increase from rest included around region). In both groups, $n=12$ at rest and $n=11$ during exercise.

Figure 5.5 Correlations in PH rats.





Correlations in PH rats between $\dot{V}O_{2\max}$ and diaphragm blood flow ($\dot{Q}$) during submaximal exercise (A), soleus and diaphragm blood flow ($\dot{Q}$) during submaximal exercise (B), and [lactate] and $\dot{V}O_{2\max}$ (C).

Chapter 6 - Conclusion

The findings of this dissertation have contributed to the field of exercise intolerance and muscle dysfunction in PH by identifying impairments in oxygen delivery, specifically the altered distribution of muscle blood flow both systemically and within respiratory muscle in PH. In resting skeletal muscle, we have demonstrated that blood flow is reduced by both convective and diffusive mechanisms, as measured using red blood cell dynamics through the capillary bed via intravital microscopy. In respiratory muscle, our results support altered regional diaphragm blood flow distribution during contractions in the anesthetized PH rat, shifting toward less mechanically advantageous regions and that this may be related to blunted vascular responsiveness in the medial costal diaphragm. Such diaphragm blood flow redistribution is also present during submaximal exercise, likely contributing to diaphragmatic weakness and dyspnea. Furthermore, diaphragm blood flow is heightened during submaximal exercise in PH compared with healthy, which may serve to “steal” blood flow from locomotory muscles, underlying the lower muscle oxygenation and contractile impairments in PH. The heightened diaphragmatic blood flow during exercise is related to impaired exercise capacity, measured using $\dot{V}O_{2\max}$, in PH rats. The collective results of these studies elucidate that skeletal muscle blood flow is lower in PH rats, which may be due to the greater ventilatory demand and diaphragmatic work commanding a higher proportion of cardiac output. The vascular dysfunction within the diaphragm, and thus regional blood flow redistribution, may contribute to the increased work and eventual failure of respiratory muscles in PH. Future investigations may target the diaphragm vasculature using pharmacological and lifestyle interventions that will aim to improve exercise tolerance and quality of life for PH patients.

Appendix A - Curriculum Vitae

EDUCATION

- 2020-2024 Ph.D. Kansas State University *Concentration: Kinesiology*
Dissertation: Skeletal and respiratory muscle oxygen transport in pulmonary hypertension
- 2020 M.S. Kansas State University *Concentration: Kinesiology*
Thesis: Nitric oxide donor reveals alterations in interstitial oxygen pressures during skeletal muscle contractions in rats with pulmonary hypertension
- 2019 B.S. Kansas State University *Major: Kinesiology*

PROFESSIONAL EXPERIENCE

- 2023-Present
Manhattan, KS **NIH NHLBI Predoctoral Research Fellow**
Cardiopulmonary Exercise Physiology Laboratory
Departments of Kinesiology and Anatomy & Physiology
Kansas State University
- 2019-2023
Manhattan, KS **Graduate Research & Teaching Assistant**
Anatomy and Physiology, Biomechanics, Biobehavioral Basis of
Physical Activity, Physiology of Exercise
Department of Kinesiology
Kansas State University
- 2018-2019
Manhattan, KS **Undergraduate Research Assistant**
Cardiopulmonary Exercise Physiology Laboratory
College of Veterinary Medicine
Departments of Kinesiology and Anatomy & Physiology
Kansas State University
- 2018-2019
Manhattan, KS **Academic Tutor**
Anatomy and Physiology, Biomechanics, Biobehavioral Basis of
Physical Activity, Measurement and Research Techniques in
Kinesiology, Physiology of Exercise
Academic Achievement Center
Kansas State University

RESEARCH INTERESTS

The Laboratory of Cardiorespiratory Physiology focuses upon investigating mechanisms responsible for altered blood flow and oxygen delivery under physiological and pathophysiological conditions.

My specific research interests include determining:

- Mechanisms to enhance peripheral vascular function and improve exercise intolerance in pulmonary hypertension.
- The effects of pulmonary hypertension on blood flow distribution at rest and during exercise, skeletal and respiratory muscle vascular dysfunction, convective and diffusive oxygen transport, fiber type and oxidative capacity of skeletal muscle.

These research aims use several methodologies including treadmill running to determine $\dot{V}O_{2\max}$ and critical speed, fluorescent microspheres to establish cardiac output and blood flow distribution at rest and during exercise, isolated microvessel techniques, phosphorescence quenching, intravital microscopy, Western blotting, and echocardiography.

RESEARCH EXPERIENCE

Visiting Scientist, Loma Linda University – May 2022

Assisted Drs. Mike Delp and Payal Ghosh in a collaborative study with NASA (RR-18) investigating the effects of gravity re-adaptations on jugular vein vasomotor function. While assisting in this project, I ran several dose-response isolated vessel experiments and was able to work with and learn from specialists in this technique.

EXTRAMURAL FUNDING (*Awarded*)

2023-2026 Ruth L. Kirschstein Predoctoral Individual National Research Service Award
National Institutes of Health – National Heart, Lung, and Blood Institute
Role for Nrf2 and exercise in mitigating pulmonary hypertension-induced vascular dysfunction.
Award number: 1F31HL170643-02 Impact Score: 33 Percentile: 33rd

PROFESSIONAL SOCIETIES

2019-present	American Physiological Society
2022-2023	Radiation Research Society
2023-present	American College of Sports Medicine

2023-present Alzheimer's Association International Society to Advance Alzheimer's Research and Treatment (ISTAART)

HONORS AND AWARDS

2024	University Distinguished Professors Excellence in Doctoral Studies (\$2600)
2024	Most Outstanding Doctoral Student, Dept. of Kinesiology, Kansas State University (\$750)
2024	Respiration Section Trainee Poster Presentation Award, American Physiological Society, American Physiology Summit Conference (\$100)
2023	Most Outstanding Graduate Teaching Assistant, Dept. of Kinesiology, Kansas State University (\$500)
2022	Best Creative Poster Award, Kansas State University Research, Scholarly and Creative Activities, and Design Forum (\$100)
2022	Cardiovascular Section Research Recognition Award, American Physiological Society, Experimental Biology Conference (\$500)
2022	Respiration Section Research Recognition Award, American Physiological Society, Experimental Biology Conference (\$500) (<i>declined</i>)
2022	2 nd Place in 3 Minute Thesis Competition, Kansas State University (\$250)
2021	Environmental & Exercise Physiology Section Gatorade Sport Science Institute Predoctoral Research Award, American Physiological Society, Experimental Biology Conference (\$600)
2021	1 st Place in Biological Sciences Oral Presentations, K-State Graduates Research, Arts, and Discovery Forum (\$500)
2021	Outstanding Teaching Award, Dept. of Kinesiology, College of Health and Human Sciences, Kansas State University (\$0)
2021	Research and Creativity Scholarship, Kansas State University (\$425)
2021	MAGS Distinguished Thesis University Representative, Biological Sciences, Kansas State University
2020	Poster Presentation Award, American Physiological Society, Integrative Physiology of Exercise Conference (\$500)

BIBLIOGRAPHY (8 first author, 2 correspondences)

1. **Schulze KM**, Hirai DM, Colburn TD, Craig JC, Musch TI, Poole DC. K_{ATP} Channel Inhibition-induced Hyporemia in Skeletal Muscle: No Evidence for Pre-capillary Sphincter Action. Under Review.
2. Scheuermann BC, Nichol K, Levenson C, **Schulze KM**, Veid C, Woodman C, Prisby R, Reyes R, Evanson K, Kuo L, Ade CJ, Muller-Delp J. Effects of aging and exercise training on cerebrovascular vasomotor reactivity, depressive behavior, and the hippocampal transcriptome. Under Review.
3. Poole DC & **Schulze KM**. Favouring Respiration over Locomotion in Chronic Obstructive Pulmonary Disease. Viewpoint on: Hartmann et al. *Experimental Physiology*. 2025.
4. Horn AG, White ZJ, Hall SE, Morrison KH, **Schulze KM**, Muller-Delp J, Poole DC, Behnke BJ. Aging impairs vasomotor function in diaphragm arterioles from male and female Fischer-344 rats. *The Journal of Physiology*. 2025.
5. **Schulze KM**, Horn AG, Weber RE, Hageman KS, Scheuermann BC, Ade CJ, Behnke BJ, Poole DC, Musch TI. Bulk and regional diaphragm blood flow during chemical hyperpnea in pulmonary hypertensive rats. *Respiratory Physiology and Neurobiology*. 2025.
6. Weber RE, **Schulze KM**, Kenney NJ, Scheuermann BC, Kunkel ON, Ade CJ, Musch TI, Behnke BJ, Poole DC. Tumor bearing in untreated breast cancer decreases exercise tolerance without lowering maximal oxygen uptake in rats. *American Journal of Cancer Research*. 2025.
7. **Schulze KM**, Horn AG, Weber RE, Kenney NJ, Hageman KS, Behnke BJ, Poole DC, Musch TI. Skeletal and respiratory muscle blood flow redistribution during submaximal exercise in pulmonary hypertensive rats. *The Journal of Physiology*. 2024
8. Horn AG, **Schulze KM**, Muller-Delp JM, Poole DC, Behnke BJ. Effects of aging on diaphragm hyperemia and blood flow distribution in male and female Fischer 344 rats. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*. 2024.
9. **Schulze KM**, Horn AG, Muller-Delp JM, White ZJ, Hall SE, Medarev SL, Weber RE, Poole DC, Musch TI, Behnke BJ. Pulmonary hypertension impairs vasomotor function in rat diaphragm arterioles. *Microvascular Research*. 2024.
10. **Schulze KM**, Horn AG, Weber RE, Behnke BJ, Poole DC, Musch TI. Pulmonary hypertension alters blood flow distribution and impairs the hyperemic response in the rat diaphragm. *Frontiers in Physiology*. 2023.
11. Scheuermann BC, Parr SK, **Schulze KM**, Kunkel ON, Turpin VRG, Liang J, and Ade CJ. Associations of cerebrovascular regulation and arterial stiffness with cerebral small vessel

disease: A systematic review and meta-analysis. *Journal of the American Heart Association*. 2023.

12. **Schulze KM** & Musch TI. Letter to the Editor. Response to: "Skeletal muscle blood flow during exercise is reduced in a rat model of pulmonary hypertension." *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*. 2023.
13. Horn AG, Kunkel ON, **Schulze KM**, Baumfalk DR, Weber RE, Poole DC, & Behnke BJ. Supplemental oxygen administration during mechanical ventilation reduces diaphragm blood flow and oxygen delivery. *Journal of Applied Physiology*. 2022.
14. **Schulze KM**, Weber RE, Horn AG, Colburn TD, Ade CJ, Much TI, Poole DC. Effects of Pulmonary Hypertension on Microcirculatory Hemodynamics in Rat Skeletal Muscle. *Microvascular Research*. 2022.
15. Weber RE, **Schulze KM**, Colburn TD, Horn AG, Hageman KS, Ade CJ, Hall SE, Sandner P, Musch TI, Poole DC. Capillary Hemodynamics and Contracting Skeletal Muscle Oxygen Pressures in Male Rats with Heart Failure: Impact of Soluble Guanylyl Cyclase Activator *Nitric Oxide*. 2021.
16. Horn AG, **Schulze KM**, Weber RE, Barstow TJ, Poole DC, Behnke BJ. Post-occlusive reactive hyperemia and skeletal muscle capillary hemodynamics. *Microvascular Research*. 2021.
17. Horn AG, Kunkel ON, Baumfalk DR, Simon ME, **Schulze KM**, Hsu W, Muller-Delp J, Poole DC, Behnke BJ. Prolonged mechanical ventilation increases diaphragm arteriole circumferential stretch without changes in stress/stretch: Implications for the pathogenesis of ventilator-induced diaphragm dysfunction. *Microcirculation*. 2021.
18. **Schulze KM**, Weber RE, Colburn TD, Horn AG, Ade CJ, Poole DC, Musch TI. The effects of pulmonary hypertension on skeletal muscle oxygen pressures in contracting rat spinotrapezius muscle. *Experimental Physiology*. 2021.
19. Ade CJ, Turpin V, Parr SK, Hammond, ST, White Z, Weber RE, **Schulze KM**, Colburn TD, Poole DC. Does Wearing a Facemask Decrease Arterial Blood Oxygenation and Impair Exercise Tolerance? *Respiratory Physiology & Neurobiology*. 2021.
20. Colburn TD, Weber RE, **Schulze KM**, Hageman KS, Behnke BJ, Musch TI, Poole DC. Sexual Dimorphism in Vascular ATP-sensitive K⁺ Channel Function Supporting Interstitial PO₂ via Convective and/or Diffusive O₂ Transport. *The Journal of Physiology*. 2021.
21. 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. *Journal of Applied Physiology*. 2020.

22. Colburn TD, Weber RE, Hageman KS, Caldwell JT, **Schulze KM**, Ade CJ, Behnke BJ, Poole DC, Musch TI. Vascular ATP-sensitive K⁺ channels support maximal aerobic capacity and critical speed via convective and diffusive O₂ transport. *The Journal of Physiology*. 2020.
23. Colburn TD, Hirai DM, Craig JC, Ferguson SK, Weber RE, **Schulze KM**, Behnke BJ, Musch TI, Poole DC. Transcapillary PO₂ Gradients in contracting muscles across the fibre type and oxidative continuum. *The Journal of Physiology*. 2020.
24. **Schulze KM**. *Master's Thesis*: Nitric oxide donor reveals alterations in interstitial oxygen pressures during skeletal muscle contractions in rats with pulmonary hypertension. <https://krex.k-state.edu/items/d475095d-0d58-4786-a61d-7ac56f3346a3>, 2020.

PRESENTATIONS

University Distinguished Professors Meeting (Kansas State University): Muscle oxygen transport in pulmonary hypertension. November 2024

Doctoral Dissertation: Skeletal and respiratory muscle oxygen transport in pulmonary hypertension. November 2024

Kansas State University College of Veterinary Medicine Seminar Series: Muscle Oxygen Transport Pathway in Disease: Implications for Pulmonary Hypertension and Breast Cancer. October 2024

University of Utah, School of Medicine: Cardiovascular mechanisms of respiratory and skeletal muscle dysfunction in pulmonary hypertension. September 2024

Kansas State University College of Veterinary Medicine Seminar Series: Oxygen Transport Pathway in Health & Disease. December 2023

Master's Thesis: Nitric oxide donor reveals alterations in interstitial oxygen pressures during skeletal muscle contractions in rats with pulmonary hypertension. July 2020.

Conference Presentations:

1. **American Physiology Summit Conference** (Poster): Sulforaphane Improves Vasodilatory Reactivity in Diaphragm Arterioles from Rats with Pulmonary Hypertension. April 5, 2024
2. **American College Sports Medicine Conference** (Poster): Effects of Pulmonary Hypertension on Blood Flow in the Contracting Spinotrapezius Muscle. May 31, 2023
3. **American Physiology Summit Conference** (Poster): Effects of Pulmonary Hypertension on Diaphragm Blood Flow. April 21, 2023
4. **Experimental Biology Conference** (Poster): Pulmonary Hypertension Induces Sex

Differences in Control of Contracting Rat Skeletal Muscle Oxygen Pressures. April 4, 2022

5. **Experimental Biology Conference** (Poster): Effects of Pulmonary Hypertension on Microcirculatory Hemodynamics in Rat Skeletal Muscle. April 12, 2021
6. **Integrative Physiology of Exercise Conference** (Poster): Effects of pulmonary hypertension on oxygen exchange in contracting rat skeletal muscle. November 8, 2020.

University Events:

1. Research, Scholarly and Creative Activities, and Design Forum (Poster): Antioxidant Supplementation Improves Blood Vessel Function in Respiratory Muscle. April 10, 2024
2. Research and the State (Poster): Just Keep Breathing: New Approaches to Improving Respiratory Muscle Function in Disease. October 24, 2023
3. Research, Scholarly and Creative Activities, and Design Forum (Poster): March 8, 2023
4. K-State Graduates Research, Arts, and Discovery Forum (Poster): March 8, 2023
5. Research, Scholarly and Creative Activities, and Design Forum (Poster): Pulmonary Hypertension: Beyond the Lungs. April 14, 2022 (Poster Award)
6. K-State Graduates Research, Arts, and Discovery Forum (Oral): Sex Differences in Skeletal Muscle in Pulmonary Hypertension. March 31, 2022
7. 3 Minute Thesis Competition (Oral): Pulmonary Hypertension: Not Just a Lung Disease? February 23, 2022 (2nd Place)
8. K-State Graduates Research, Arts, and Discovery Forum (Oral): Effects of Pulmonary Hypertension on Microcirculatory Hemodynamics in Rat Skeletal Muscle. March 31, 2021. (1st place)

TEACHING EXPERIENCE

Kansas State University

- KIN 220 Bio-Behavioral Basis of Exercise Laboratory (1 semester)
- KIN 330 Biomechanics Laboratory (2 semesters)
- KIN 336 Physiology of Exercise Laboratory (4 semesters)
- KIN 360 Anatomy and Physiology Laboratory (8 semesters)
- Anatomy and Physiology Interim Lab Coordinator (1 semester)
- Guest lectures for Anatomy and Physiology, Physiology of Exercise

TEACHING EXCELLENCE (Fall 2019-Spring 2023)

TEVAL scores for overall effectiveness as an instructor (adjusted average on scale of 5.0):

Anatomy and Physiology (4.8), Exercise Physiology (4.8), Biobehavioral basis of physical activity (4.7), and Biomechanics (4.6).

EDITORIAL AND REVIEWING ACTIVITIES

Journal Review:

- *Journal of Thoracic Disease*
- *Acta Biochimica et Biophysica Sinica*
- *Experimental Physiology*
- *American Physiological Society* journals
- *BMC Medicine*

ACADEMIC AND PROFESSIONAL SERVICE

2022-2024	Department of Kinesiology Social Media and Marketing Committee ▪ Graduate Student Coordinator
2023	APS Peer-Review Certification
2021-2023	Graduate Student Council Leadership Team ▪ Recreation Services Representative ▪ Student Affairs Committee Member
2021-2022	College of Veterinary Medicine Graduate Student Association Executive Team ▪ Veterinary Medicine-Kinesiology Department Liaison

MENTORING AND TRAINING

Tyler McCoach, M.S. (Spring 2022-Fall 2023) – *University of Kansas Medical Student*

Cameron Heying, B.S. (Spring 2022-Spring 2023) – *Applying to medical programs*

Hannah Wall, B.S. (Summer 2022) – *Applying to medical programs*

Nathan Kenney, B.S. (Fall 2022-Current) – *Graduate student at Kansas State University*

McKinley Reagor (Summer 2024) – *Kansas Idea Network of Biomedical Research Excellence (K-INBRE) visiting summer scholar*

Alauna Parker (Summer 2024) – *Kansas Idea Network of Biomedical Research Excellence (K-INBRE) visiting summer scholar*

Liz Wolfram (Fall 2024) – *International visiting scholar*

Daniel McVay (Fall 2024-Spring 2025) – *Undergraduate research*

SCIENTIFIC ABSTRACTS (7 first author, 17 co-author, 24 total)

1. **Schulze KM**, Horn AG, Weber RE, Kenney NJ, Muller-Delp JM, Behnke BJ, Poole DC,

- Musch TI (2024). Sulforaphane improves vasodilatory reactivity in diaphragm arterioles from rats with pulmonary hypertension. *Physiology*.
2. Weber RE, Kunkel OK, **Schulze KM**, Kenney NJ, Hageman KS, Horn AG, Musch TI, Behnke BJ, Poole DC. (2024). Tumor oxygenation in an orthotopic model of breast cancer: impact of dietary nitrate supplementation. *Physiology*.
 3. Horn AG, **Schulze KM**, Weber RE, Kunkel OK, Poole DC, Behnke BJ. (2024). The impact of aging and biological sex on diaphragm hyperemia and blood flow distribution. *Physiology*.
 4. Kenney NJ, Horn AG, **Schulze KM**, Weber RE, Poole DC, Behnke BJ, Musch TI. (2024). Effects of aging on vasoconstrictor responses of diaphragm arterioles. *Physiology*.
 5. Sudasinghe L, White ZJ, Rogers LG, Veith S, Wang W, **Schulze KM**, Hall SE. (2024). Intermittent fasting alters O₂ consumption and body composition in young rats: comparative analysis between males and females. *Physiology*.
 6. **Schulze KM**, Horn AG, Weber RE, Ade CJ, Behnke BJ, Poole DC, & Musch TI. (2023). Effects of Pulmonary Hypertension on Blood Flow in the Contracting Spinotrapezius Muscle. *Medicine & Science in Sports & Exercise*.
 7. Horn AG, **Schulze KM**, Kunkel ON, Poole DC, & Behnke BJ. (2023). Effect of Angiotensin-(1-7) on Diaphragm Blood Flow during Mechanical Ventilation. *Medicine & Science in Sports & Exercise*.
 8. Weber RE, **Schulze KM**, Hageman KS, Musch TI, & Poole DC. (2023). Nitric Oxide-soluble Guanylyl Cyclase Activator Increases Endurance Capacity and Skeletal Muscle Oxygenation in Male Rats. *Medicine & Science in Sports & Exercise*
 9. Rogers LG, Hall SE, AE Pahua, C King, **Schulze KM**, Plakke B. (2023) Effect of Treadmill Training in a Rodent Model of Autism Spectrum Disorder. *Medicine & Science in Sports & Exercise*
 10. **Schulze KM**, Horn AG, Weber RE, Ade CJ, Behnke BJ, Poole DC, & Musch TI. (2023). Effect of Pulmonary Hypertension on Diaphragm Blood Flow. *Physiology*, 38.
 11. Weber RE, **Schulze KM**, Hageman KS, Musch TI, & Poole DC. (2023). Increased Nitric Oxide-Soluble Guanylyl Cyclase Signaling Improves Endurance Capacity and Contracting Skeletal Muscle Oxygenation in Healthy Female Rats. *Physiology*, 38.
 12. Horn AG, Kunkel ON, **Schulze KM**, Weber RE, Poole DC, & Behnke BJ. (2023). The Impact of Aging and Sex on Diaphragm Vasomotor Function. *Physiology*, 38.
 13. **Schulze KM**, Weber RE, Horn AG, Colburn TD, Ade CJ, Poole DC, & Musch TI. (2022). Pulmonary Hypertension Induces Sex Differences in Control of Contracting Rat Skeletal Muscle Oxygen Pressures. *The FASEB Journal*, 36.

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