THE EQUINE PULMONARY MICROVASCULATURE AND ITS POTENTIAL ROLE IN EXERCISE-INDUCED PULMONARY HEMORRHAGE

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Comparative Medicine and Integrative Biology – Doctor of Philosophy

2014

ABSTRACT

STUDY OF THE EQUINE PULMONARY MICROVASCULATURE AND ITS ROLE IN EXERCISE-INDUCED PULMONARY HEMORRHAGE

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Exercise-induced pulmonary hemorrhage (EIPH) is diagnosed by the presence of frank blood in the airways following a bout of intense exercise. EIPH affects all racehorses, and has been diagnosed in other athletic species, including humans. EIPH is associated with impaired racing performance and significant pulmonary pathology in the caudodorsal lung, while the cranioventral lung is spared. Lesions include hemosiderin accumulation, interstitial and septal fibrosis, angiogenesis, capillary wall disruption and remodeling of small-caliber (100 – 200 μm diameter) intralobular pulmonary veins. High pressures in the pulmonary circulation of the exercising horse cause capillary stress failure, resulting in the main symptom: hemorrhage. Stress failure alone does not account for all EIPH lesions, and in particular, venous remodeling. Nor does it explain regional predilection of EIPH pathology. EIPH pathogenesis awaits complete explanation at this time. Capillary pressure is determined in part by resistance to flow in the arteries and veins that supply and drain a capillary bed. Decreased arterial, and increased venous resistance are conditions under which capillary pressures will increase, and may approach arterial pressure values.

I hypothesize that a combination of increased blood flow during exercise to caudodorsal lung, coupled with exercise-associated alterations in vessel tone provides transient but sufficient hemodynamic stimuli to initiate venous remodeling in this region only. Therefore the impact of ongoing venous remodeling would be to reduce venous wall compliance, thereby increasing venous resistance to flow and increasing capillary

pressures in the caudodorsal lung. Capillary wall stress failure, hemorrhage, and EIPH result.

The characteristics of small vessels that determine capillary pressure include mechanical and reactivity profiles of small arteries and veins that work to effect passive and active changes in vessel diameter, along with any impact of remodeling on venous wall compliance. These were evaluated in three studies. The important findings are as follows:

First, regional differences in mechanical properties of arteries and veins exist in control, unraced horses, which may reflect inhomogeneous blood flow distribution in the equine lung. Racing is associated with increased stiffness of caudodorsal pulmonary veins only, despite the absence of severe EIPH pathology.

Second, autonomic control of small, equine pulmonary arteries and veins is not consistent across lung regions, and the reported differences, when extrapolated to *in vivo* exercising conditions of increased sympathetic input, can account for increased capillary pressure in caudodorsal lung.

Finally, although exercise causes significant alterations in mRNA expression in vein walls, the changes do not support initiation of a remodeling response after only 2 weeks of intense exercise.

These data contribute to the understanding of EIPH pathogenesis, and highlight the pivotal role of the pulmonary microvasculature, in particular the pulmonary veins, in this significant, ubiquitous disease. Furthermore, regional differences in mechanical properties and reactivity profiles of pulmonary vessels of this caliber are not reported in any other species, and as such, these findings may have farther-reaching applications in the field of pulmonary vascular biology.

This dissertation is dedicated to Geoff,
in recognition of the many thousands of miles he traveled.

ACKNKOWLEDGEMENTS

I would like to express my sincere gratitude to my advisor Dr. Frederik J. Derksen for his unfaltering enthusiasm, mentorship and support.

I also wish to acknowledge and thank the following members of my doctoral committee – Dr. William F. Jackson, Dr. Kurt J. Williams and Dr. N. Edward Robinson for their guidance and valuable advice.

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KEY TO ABBREVIATIONS

BAL	Broncho-alveolar lavage
CD	Caudodorsal
CV	Cranioventral
EIPH	Exercise-induced pulmonary hemorrhage
ET	Endothelin
HR_{max}	Maximum heart rate
MMP	Matrix metalloproteinase
O.D.	Outer diameter
P_{cap}	Pulmonary capillary pressure
P_{la}	Left atrial pressure
P_{pa}	Pulmonary arterial pressure
P_{tm}	Pulmonary transmural pressure
P_{wp}	Pulmonary wedge pressure
PDGF	Platelet-derived growth factor
PVR	Pulmonary vascular resistance
qRT-PCR	Quantitative real-time PCR
TGF-β	Transforming growth factor-beta
TIMP	Tissue inhibitor of metalloproteinase
VEGF	Vascular endothelial growth factor

CHAPTER 1

Literature Review

Section 1: Exercise-induced pulmonary hemorrhage

This section is designed to provide comprehensive background information on exercise-induced pulmonary hemorrhage (EIPH). Specifically, deficits in our understanding of pathogenic mechanisms will be addressed, thereby providing justification for further study of EIPH pathogenesis.

History

Exercise-induced pulmonary hemorrhage (EIPH) is defined as the presence of blood in the airways after an intense bout of exercise, and is a ubiquitous, performance-limiting condition of modern-day racehorses. Post-exercise epistaxis has been recognized for hundreds of years however.

Markham's Masterpiece containing "all knowledge touching the curing of all diseases of horses" that was first published in 1610, has a chapter dedicated to the topic.

"*Many horses (especially young horses) are often subject to this bleeding at the nose, which I imagine proceedeth either from the abundance of Blood, or that the Vein which endeth in that Place is either broken, fretted, or opened*...

...it (the Vein) may be broken by some violent strain" (130)

The Thoroughbred stallion Bartlet's Childers was born in 1716 and was also known as "Bleeding Childers" in his younger years. The horse was never raced, which according to racing lore, was due to severe EIPH. He was full brother to Flying Childers, who is

described in the General Stud Book as "the fleetest horse that was ever trained"(1). Despite being unraced, Bartlet's Childers was bred extensively, and "got so many good horses, that he is ranked with the first-rate stallions" (1). Indeed, he is the great-grandsire of Eclipse – a hugely influential stallion to whom more than 95% of English Thoroughbreds are genetically linked (221).

In his publication "Epistaxis in the Racehorse" published in 1974, Cook suspects that the epistaxis observed after exercise is from a pulmonary source, as he used a rigid endoscope to rule out upper airway (rostral to the larynx) sources of hemorrhage(32). The term "exercise-induced pulmonary hemorrhage" was first used in 1981 when, through use of a flexible endoscope, Pascoe *et al* confirmed that post-exercise epistaxis in horses was in fact of pulmonary origin (162).

Epidemiology

It is worth noting that EIPH is not limited to racehorses. EIPH has been described in horses that participate in disciplines other than racing, such as polo (220), rodeo (8), and also in racing greyhounds (42), and camels (4). There are also reports of EIPH in small numbers of (mostly elite) human athletes, specifically after running (52), cycling (79) and swimming (227). Due to an absence of routine post-exertion diagnostics, the true prevalence of EIPH in non-racing horses, and in other species is not known. However in all reports, the level of exercise resulting in EIPH can be considered "intense."

If epistaxis is used as the diagnostic criterion, reported prevalence of EIPH in Thoroughbreds is actually low. For example, in Thoroughbreds racing in South Africa, and Japan, the epistaxis rate is less than 0.2 % (205, 226). When endoscopy of the upper

airways including the trachea, is performed within 2 hours of racing, reported EIPH prevalence is much higher. Based on a single post race endoscopic examination, between 62 and 75 % of racing Thoroughbreds, Standardbreds and Quarterhorses experience EIPH (18, 68, 107, 172). When horses are evaluated after multiple races, it is reported that 87% to 100% of Thoroughbreds and Standardbreds have evidence of EIPH after one of three races (18, 107).

Post-exercise broncho-alveolar lavage (BAL), which can be performed up to a number of weeks after an exercise bout, is also used to diagnose EIPH based on the presence of free red blood cells and hemosiderin-laden macrophages in lavage fluid. Using BAL as the diagnostic technique, EIPH prevalence in Thoroughbreds approaches 100% (131).

Risk factors for EIPH

Regardless of species, or the type of racing, EIPH is typically associated with intense exercise. Although underlying pathology, for example atrial fibrillation, may increase an individual animal's risk of experiencing EIPH (36), this is not a common, concurrent clinical finding in horses with EIPH.

Under normal circumstances, numerous risk factors have been purported to contribute to EIPH risk. Horses are at increased risk of EIPH that manifests as epistaxis when they race over fences (versus flat racing), and when they are older (145, 205). When risk factors associated with endoscopically-diagnosed EIPH are evaluated, it transpires that increased EIPH risk associated with increasing age is probably due to a higher number of

race-starts (and presumably days in training) in older animals compared to younger ones (75).

Clinical features

Other than epistaxis (a relatively rare occurrence), and anecdotal reports of increased swallowing post-exercise, EIPH is not associated with clinically detectable symptoms. However it has long been believed that EIPH is associated with compromised racing performance, and this was demonstrated conclusively in 2005 based on evaluation of a population of racehorses in Australia. This report employs an endoscopic scoring system which has been widely adopted as standard, and demonstrated to have excellent inter-observer reliability (69). Using this system, horses with no blood in the trachea are assigned grade 0, and horses with blood covering >90% of the tracheal surface are assigned grade 4. Horses with mild (grade 1) or no EIPH are 4 times as likely to win and almost twice as likely to place in a race compared to horses with EIPH grade 2, 3, or 4 (70).

Pathology

A series of publications by O'Callaghan *et al,* contain some of the first descriptions of EIPH-associated gross and microscopic pathology. 26 horses that were retired from racing due to severe EIPH were studied. Gross pathologic changes included dark blue-black discoloration of caudodorsal lung, with up to 45% of lung affected, and these areas were firmer than normal lung tissue (150, 151). Hemosiderin accumulation, angiogenesis (due to bronchial circulation proliferation), bronchiolitis and extensive fibrosis are also described (151, 152). These findings are consistent with a subsequent publication on the topic (157). However,

in two publications from investigators at Michigan State University that evaluate pulmonary pathology of racehorses from both Singapore and North America, bronchiolitis is not reported to be a consistently occurring lesion (231, 233). A likely reason for this discrepancy is due to the fact that inflammatory airway disease is common in young horses in training (237), and reports of small airway pathology in EIPH-affected lung actually reflect coincident disease processes whose pathogeneses are unrelated.

A novel lesion - that of remodeling of small intra-lobular pulmonary veins is described in the caudodorsal lung tissue of both the Singapore and North American cohorts however (231, 233). Remodeling affects pulmonary veins up to approximately 200 μm outer diameter (O.D.) and is characterized by expansion of the adventitia by mature collagen in affected vessel walls. Collagen is also found between the external elastic lamina and the vein lumen. In some veins, *tunica media* and *tunica intima* hypertrophy is also observed, and in severely-affected vessels, luminal area appears markedly reduced (231). Morphometric analyses support these observations. In lung tissue from EIPH-affected horses, veins are significantly thicker-walled in dorsal lung and walls are thickest in the most caudodorsal region (38). Veins also have reduced luminal area in the most severely affected lung regions, compared to vessels from less affected, or normal lung tissue (38).

A recent detailed, systematic evaluation of EIPH-affected lungs supplies more information about venous remodeling in the context of other EIPH pathologic features. Venous remodeling, hemosiderin accumulation and fibrosis are more common, and most severe in caudodorsal lung, compared to cranial and ventral lung (233). In 93% of 1,400 randomly selected samples, venous remodeling was present with hemosiderin, and in the remaining samples, venous remodeling was mild. Also, interstitial fibrosis was rarely

present (0.3% of samples only) without venous remodeling. These data strongly implicate venous remodeling as a lesion of interest in EIPH, and likely one that occurs early in the disease process, and is central to EIPH pathogenesis.

Pathogenesis

A number of theories of EIPH pathogenesis have been proposed and include some that implicate airway inflammation as a cause (153, 178), and another that attributes hemorrhage to lung trauma resulting from transmission of a shock wave from the ground to the lung during galloping (184). However neither of these theories can adequately explain the constellation of lesions that are associated with EIPH, nor the distinct caudodorsal distribution of EIPH pathology.

In the early 1990s it was reported that mean pulmonary artery pressure in the galloping horse was in the order of 90 – 110 mmHg, and that pulmonary wedge pressures and left atrial pressures were 56 and 70 mmHg respectively (88, 124). These values deliver estimated intravascular capillary pressures of 72 – 83 mmHg (124, 127). At the same time, a breakthrough publication by West *et al* described pulmonary capillary wall disruption in lung tissue from horses that had recently exercised on a treadmill. Extravascular red cells were found both in the pulmonary interstitium and in surrounding alveolar airspaces (230). These data suggest that the pulmonary circulation is the source of airway hemorrhage, and the authors went on to propose that capillary rupture was secondary to the high pulmonary capillary pressures during exercise (229). Further support for the capillary stress-failure theory was provided by Birks *et al* who demonstrated that the estimated threshold for breaking strength of equine pulmonary capillaries was exceeded at

a value of 75 mmHg transmural pressure (P_{tm}) (17). This value in the horse is higher than that of rabbit or dog pulmonary capillaries (16), and based on multiple studies, falls within the range of estimated pulmonary capillary pressures during exercise (124, 127) particularly when negative alveolar pressure (a component of P_{tm}) is taken into account (106).

As a point of interest, the reason that the extremely thin-walled, and apparently fragile capillary can withstand very high intravascular pressures (up to a certain point) is not due to the inherent strength of its wall, but rather its very small diameter (5 – 8 μm) (210), as predicted by the Laplace relationship between wall tension (T), intraluminal pressure (P) and vessel radius (r)

$$T = P \times r$$

At normal, resting, pulmonary capillary pressures, the tension that must be maintained by the capillary wall to resist distension and rupture is only 16 dynes/cm. This is approximately 3000 times *less* than the breaking strength of a piece of wet tissue paper (whose breaking strength is 50,000 dynes/cm) (20)!

Pulmonary capillary pressure is determined in part by the resistance to flow in the arteries and veins that supply and drain the capillaries. In turn, resistance to flow is strongly influenced by vessel diameter. Vessel diameter is determined by the vessel wall structure (a so-called passive factor) that acts to resist distension, and vascular reactivity (an active factor), which is controlled by smooth muscle contraction/relaxation (11). Increased resistance in the venous compartment, and/or decreased resistance in the arterial compartment are both conditions under which pulmonary capillary pressure will increase, and potentially approach arterial values. Regional differences in pulmonary vessel

reactivity are reported in large-caliber pulmonary arteries in the pig, and the horse (164, 176). Should regional differences in the determinants of small arterial and venous diameter exist in a pattern that causes highest pulmonary capillary pressures in caudodorsal lung, this could provide a reason for the predilection of pathology for this lung region.

A study using microspheres demonstrated that pulmonary blood flow distribution in the horse is not gravitationally dependent. Blood flow is highest in dorsal regions, compared to ventral ones, and in caudal lung compared to cranial (76). Furthermore, blood flow within isogravitational planes displays significant heterogeneity (average coefficient of variation 30.7%). A recent publication from our laboratory demonstrated that EIPH pathology (venous remodeling, hemosiderin and interstitial fibrosis) distribution matches that of pulmonary blood flow (233). It is also worth noting that exercise does not change the overall pattern of blood flow, but does result in yet further increased flow to dorsal lung (15), while sparing ventral lung. A possible reason for redistribution of blood to this region during exercise include regional differences in vascular reactivity, which has been demonstrated in large (6 mm O.D.) equine pulmonary arteries (164).

That EIPH lesion distribution matches blood flow distribution (233) is particularly interesting when venous remodeling pathogenesis is considered. Vascular remodeling, and specifically pulmonary venous remodeling is a classic adaptive response to increased intravascular flow and/or pressure (64, 86). The region of lungs in which veins remodel in EPIH is the region that also receives the highest flow during exercise (15). Remodeled veins have increased wall collagen content and reduced lumen diameter (38). It is likely that these changes decrease venous wall compliance, and may effectively impede capillary drainage and increase pulmonary capillary pressure as a result.

Whether EIPH pathology can be reproduced by the presence of blood in the airway (i.e. without exercise) has been investigated. Neither a single nor repeated instillations of autologous blood into the small airways resulted in reproduction of EIPH-associated interstitial fibrosis (39, 232). A reasonable interpretation of these data is that capillary stress failure and presumably, interstitial hemorrhage are required for development of the fibrotic component of EIPH pathology. Simple capillary stress failure alone does not take into account the distinctive regional pattern of the EIPH lesion however.

Taking all of this information into account led to the development of a more detailed theory of pathogenesis, which follows:

During intense exercise horses experience elevated pulmonary artery, left atrial and pulmonary capillary pressures. In caudodorsal regions of lung that already experience highest flow, regional differences in determinants of arterial and venous vessel diameter promote even higher pulmonary capillary pressures. Pulmonary capillary breaking strength is exceeded resulting in stress failure of some capillaries, and extravasation of red cells and airway hemorrhage. During both training and racing, repeated episodes of high pulmonary blood flow and pressures, particularly in the highest flow regions within caudodorsal lung, result in pulmonary venous remodeling. Remodeled pulmonary veins are less compliant than normal veins and failure of these vessels to distend normally further increases pulmonary capillary pressures, which in turn augments stress-failure and hemorrhage. The increased risk of EIPH for horses with more race starts is a reflection of ongoing venous pathology that results from transient but recurrent exercise-associated pulmonary hypertension.

It's clear that there remain gaps in our understanding of EIPH pathogenesis however, and my outlined theory of pathogenesis requires more detailed investigations to provide corroboration for all of its components, in particular regional control of pulmonary capillary pressure, and the mechanisms and physiologic ramifications of venous remodeling. The overarching aim of this dissertation therefore is to further investigate the equine pulmonary microvasculature in order to better elucidate EIPH pathogenic mechanisms.

To reach this goal I investigated the effect of exercise on pulmonary veins specifically, as these vessels are remodeled in EIPH-affected lung and I hypothesize that this remodeling affects venous compliance. Vein wall gene and protein expression, and pulmonary venous mechanical characteristics after exercise are evaluated in studies 1 and 3.

I also evaluated the mechanisms that control small pulmonary artery and vein tone in the horse lung, and specifically whether or not these mechanisms exhibit a regionally heterogeneous pattern. The rationale for this component is that these vessels directly impact pulmonary capillary pressure. Furthermore, EIPH pathology has a distinct regional distribution. In order to understand EIPH pathogenesis, our understanding of how the tone of these vessels is regulated during exercise, and whether regional differences in these control mechanisms needs to be investigated.

Treatment

Many pharmacologic agents have been used to treat EIPH, for the most part in the absence of proof of efficacy. For the purposes of this summary, discussion will be limited to the most widely used therapy, and the only one with evidence to support its use, furosemide.

Furosemide (4-chloro-N-2[(furylmethyl)amino]-5-sulfamoylbenzoic acid) is a high-ceiling or loop diuretic that is licensed in the United States for use in horses for the treatment of edema (e.g. pulmonary congestion, ascites) associated with cardiac insufficiency, and acute non-inflammatory tissue edema (http://www.accessdata.fda.gov/scripts/animaldrugsatfda/details.cfm?dn=034-478). Furosemide's use as a pre-race treatment in horses became commonplace in the 1970s, and the drug is currently used in excess of 90% of race starts in North America (74). High quality evidence of its efficacy in EIPH treatment was finally provided in 2009 when results of a blinded, placebo-controlled, crossover study of 167 racehorses were published. Horses that were treated with a saline placebo before racing were approximately 4 times as likely to develop EIPH than those horses that received furosemide (74). Furosemide did not prevent EIPH in this population, but almost 70% of horses that received furosemide and experienced EIPH had a reduced severity score of one or more grades (69) compared to the placebo treatment arm (74).

Furosemide is associated with enhanced racing performance in both Thoroughbred and Standardbred horses (60, 192), and while it is proposed by many that this is due to its amelioration of performance-limiting EIPH, data that can prove this association have not been published to date.

The mechanism of action of furosemide in EIPH is attributed to its diuretic effect, and the resulting 8% reduction in plasma volume (72). This is turn is associated with a rapid and

sustained decrease in right atrial pulmonary artery pressure (71, 158). Pulmonary wedge and capillary pressures are also reduced by approximately 20 % during exercise after treatment with furosemide (54, 123). As these effects are abolished when post-furosemide fluid losses are replaced with intravenous fluids before exercise (71), the effect of furosemide on EIPH is probably due in large part to attenuation of intravascular pulmonary pressures during exercise. However, it is reported by some that cardiac output during exercise is unaffected by furosemide administration, and furthermore, that pulmonary blood flow distribution is altered in a manner that spares caudodorsal lung (43). In the face of similar cardiac output, altered blood flow distribution has to be attributed to changes in vascular reactivity. Furosemide is reported to be a systemic and pulmonary venodilator in humans and dogs respectively (59, 170). I propose therefore the effect of furosemide on EIPH is attributable at least in part to its action on pulmonary veins, and not solely to plasma volume reduction. This is investigated in the second study described in this dissertation.

Summary

EIPH is a highly prevalent condition of a large population of athletic horses, which limits their performance, and results in significant pulmonary pathology. In the absence of thorough understanding of underlying disease mechanisms, effective therapies and management strategies for EIPH will remain elusive.

Section 2: The pulmonary microvasculature

This section is designed to highlight why detailed study of the pulmonary microvasculature is warranted in the investigation of EIPH pathogenic mechanisms. To achieve this, vascular wall mechanical properties, and small vessel reactivity will be discussed. This will provide context and rationale for the following studies: "Lung region and racing affect mechanical properties of equine pulmonary microvasculature" and "Regional differences exist in autonomic control of equine pulmonary vascular reactivity."

The pulmonary circulation

The earliest descriptions of the pulmonary circulation are attributed to Michael Servetus (b. 1511), a French physician and theologian who was burned at the stake for heresy in 1553 (197). He referred to the pulmonary circulation as the "*vital spirit...a mixture of inhaled air and subtle blood*," and refuted the commonly held belief at the time that blood moved across the septum of the heart, instead proposing its actual route through the lungs. Servetus' reasoning is flawless, but proved very unpopular at the time.

"Many facts prove the reality of this communication of the blood through the lungs...a confirmation is provided by the huge width of the arterial vein [the pulmonary artery]. *The arterial vein itself would never have been constructed this way, nor would it be so wide, and it would not be forwarding such a powerful jet of the purest of blood from the heart to the lungs simply in order to provide nourishment for the lungs; the heart would never have placed itself in the service of the lungs in this manner."*

Needless to say, since Servetus' time, our understanding of the pulmonary circulation anatomy and function has advanced considerably. Evidence that the pulmonary circulation is the source of hemorrhage in EIPH was published in 1993 by John West and colleagues (230), and this group of investigators was the first to propose that stress-failure of pulmonary capillaries occurs secondary to the high intravascular, and specifically high capillary pressures that normally occur in the horse during exercise (229).

Elucidation of the factors that determine this capillary pressure therefore, should be considered crucial for complete understanding of EIPH pathogenesis.

Both pulmonary arterial and left atrial pressures determine pulmonary capillary pressure. Pappenheimer and Soto-Riviera determined capillary pressure in an isolated dog/cat limb by use of the isogravimetric technique, and demonstrated that it required a significantly smaller increase in venous pressure, compared with arterial pressure, to cause a given increase in filtration (i.e. pressure) across the capillary bed (160). Gaar and colleagues employed the same technique to estimate pulmonary capillary pressure, and arterial and venous resistance to flow in the isolated dog lung in 1967 (49). They determined that under normal conditions of flow (that do not cause capillary filtration), pulmonary arteries and veins contribute 56 and 44% respectively to pulmonary vascular resistance, and that capillary pressure P_{cap} can be calculated as follows:

$$P_{cap} = P_{la} + 0.4 \times (P_{pa} - P_{la})$$

where P_{la} and P_{pa} are left atrial and pulmonary artery pressures respectively (141). This equation does not hold true in conditions of high flow rates however, such as are experienced by the horse whose cardiac output increases at least 6 fold during exercise (44). As flow is increased, resistance in the arterial compartment decreases, whereas that

of the venous compartment increases. At 10 times normal flow in an isolated dog lung lobe, the (venous) compartment downstream of the capillary bed contributes 86% of pulmonary resistance to flow (238).

More recent descriptions of EIPH pathology clearly indicate that pathogenic mechanisms are more complex than simple stress-failure (231), and that pulmonary venous remodeling, whose distribution can be predicted by that of pulmonary blood flow, is also a fundamental process underpinning EIPH pathogenesis (233). As it is highly likely that venous wall remodeling increases resistance to flow above normal in a vascular compartment that already contributes the majority of pulmonary vascular resistance in high flow states, pulmonary veins in particular are considered worthy of further investigation and scrutiny in the context of EIPH.

The pulmonary circulation, and specifically the arteries and veins that supply and drain the pulmonary capillaries, therefore form the central focus of all experiments described in this dissertation.

Vessel anatomy

EIPH-associated venous remodeling is reported in equine pulmonary vessels that are between 100 and 200 μm in outer diameter (O.D.) (231). In humans, pulmonary arteries measuring less than 100 μm O.D. are (arbitrarily) classified as arterioles by some authors (the term is avoided by others (34)), and have varying amounts of smooth muscle (95). In my studies on equine pulmonary vasculature I selected muscular pulmonary arteries and veins that range between 100 and 400 μm O.D. in order to encompasses vessels within the

diameter range that are immediately upstream from pulmonary arterioles, and veins from within the diameter range that is reported to remodel.

Based on the Strahler ordering system, wherein order 1 is the smallest, noncapillary branch of the pulmonary arterial (or venous) network, the average diameter ratio across multiple species for increasing order is 1.65 ± 0.11 (210). This system is suitable for application to the pulmonary circulation due to the irregular branching pattern, and many "generations" of vessels can be attributed to the same order if their diameters are similar. Human lungs have 17 orders of pulmonary artery branches (80). Based on a starting diameter of approximately 15 μm in first order arterioles, with an increase in diameter of 65 % with each new branch order, the equine pulmonary vessels (both arteries and veins) used in these studies range between orders 5 and 7.

In order to perform experiments on small caliber pulmonary arteries and veins, accurate and reliable identification of these vessels in the pulmonary parenchyma during vessel dissection is a key technique. McLaughlin and colleagues classified the equine lung as a type III lung, based in part on their observation that the pulmonary veins did not always travel with bronchi and pulmonary arteries, tending instead to take a "more direct, independent course to the hilum" (132). Pulmonary arteries on the other hand, are generally accompanied by a paired airway, and share a common connective tissue sheath (210). These spatial descriptors provide the basic criteria for discerning small pulmonary arteries from intralobular pulmonary veins in equine lung tissue during dissection.

Histological confirmation of vessel identity was also used as a secondary identification technique in these experiments, as relying exclusively on the anatomic distribution described above may be prone to error for the following reasons. In peripheral

equine pulmonary vessels there is a tendency for pulmonary veins to be more closely associated with the bronchovascular bundle in Type III lungs (132), although in this author's experience, veins are generally discernable from arteries in such a triad, in that they are thinner-walled, and not as adherent to the airway as pulmonary arteries typically are. Also, supernumerary arteries have been described in the lungs of humans, sheep, pigs, cows and rats (41, 185). These are arteries that branch from the parent pulmonary artery at 90 degrees and travel unaccompanied by an airway through pulmonary parenchyma (210). Should these arteries exist in horses, they could be mistaken as intraparenchymal pulmonary veins based on the absence of an adjacent airway.

The relatively thick *tunica media* of muscular pulmonary arteries such as those studied is contained by both an internal and an external elastic lamina, whereas pulmonary veins possess only an external elastic lamina that separates the *tunica adventitia* from a thin *tunica media* (which contributes between 33 and 60% less to wall thickness in veins than it does in pulmonary arteries) (134, 175, 210). Staining of vessels segments in cross section with hematoxylin and eosin, and Verhoef-Van Gieson (to stain elastin specifically) was utilized to discern between arteries and veins, and confirm or refute their dissection identity based on *tunica media* thickness and number of laminae.

Vessel wall mechanical properties

As early as 1880, Charles Roy published results of experiments demonstrating that arterial walls, amongst other tissues, did not conform to Hooke's Law for elastic materials (179). In general terms, Hooke's law states that strain (deformation) of an elastic material is proportional to the stress (force) applied to it. In other words, with the application of

increasing loads, true elastic materials become more extensible. In contrast, Roy observed that arteries demonstrate increasing resistance to stretch as applied stress is increased (179).

The ability of a vessel to resist stretch is an important quality, as it is this passive tension (which can be calculated using the Laplace relationship) that counteracts the tendency of the blood pressure to distend a vessel indefinitely (or to rupture), and this maintenance passive tension can be achieved without expenditure of energy (20). The main structural components of vessel (other than capillary) walls are endothelial cells, vascular smooth muscle, elastin and collagen. The contributions of endothelial cells and vascular smooth muscle to the ability of a vessel to passively resist stretch are considered minimal (20), however the role of elastin and collagen in conferring this property on a vessel wall have been investigated in some detail. In 1957 a key manuscript published by Roach and Burton entitled "the reason for the shape of the distensibility curves of arteries" explained Roy's observations using an eloquently-designed study (177). Using human iliac arteries, these investigators generated pressure-volume curves, which were subsequently converted into tension-length curves using the Laplace relationship, from 3 sets of vessels: these comprised of control vessels, arteries that had undergone formic acid digestion to selectively remove collagen, and arteries that undergone a trypsin digestion to remove all elastin fibers. By subtraction, and comparison with control vessels, the individual contributions of elastin and collagen to the mechanical behavior of an arterial wall under stress were elucidated. In summary, it can be deduced from their data that elastin is responsible for wall tension at low pressures (i.e. the lower, flatter portion of a typical arterial length-tension curve) whereas collagen is responsible at higher pressures (i.e. in

the steeper portion of the curve) (177). At low pressures, collagen remains unstretched and effectively coiled, however with increasing pressures collagen is stretched and contributes more to vessel wall tension. Roach and Burton's data speak to the fact that the Young's (elastic) modulus of collagen is many 100-fold higher than that of elastin, indicating that the former can withstand much more stress without significant deformation than elastin can (20).

That pulmonary arteries are more distensible that pulmonary veins has been described in the rabbit (22), the dog (121) and in large pulmonary arteries and veins in people (9, 119). This difference is attributed to the greater proportion of collagen in venous compared to arterial walls (121).

Distensibility of the pulmonary circulation is a mechanical property that reflects the % change in diameter of a vessel / mmHg pressure, and is determined in large part by vessel wall structure. Distensibility is denoted by the distensibility coefficient α. Overall distensibility of the human pulmonary circulation (arteries and veins) is estimated at 0.02 (i.e. a 2% change in vessel diameter for each mmHg increase in pressure), whereas that of the horse is lower, and calculated at 0.01 (174), although these particular figures are calculated from *in vivo* pulmonary arterial and pulmonary wedge pressures, therefore the impact of changes in vessel diameter resulting from active (smooth muscle-mediated) alterations in vessel tone must be considered superimposed on distensibility determined by mechanical properties of the vessel walls. That being said, distensibility of isolated pulmonary vessels of varying size, in multiple species is also approximately 2% (102), and for the most part, this figure is a reflection of these isolated vessels' mechanical properties only. This is an interesting observation that suggests that active changes in vessel tone have

a relatively small impact on *overall* distensibility of the pulmonary circulation, and that mechanical properties of vessels are worthy of consideration in studies of pulmonary circulation hemodynamics.

It seems paradoxical that the distensibility of arteries and veins of the pulmonary circulation has been demonstrated to be relatively independent of vessel diameter in multiple species (5, 33, 94), although distensibility between vessels in same size range does differ. It is proposed by some authors that the constant nature of this parameter effectively preserves the overall distribution pattern of cardiac output to the lungs when flow is increased (102). The reason suggested for why this is a desirable characteristic is that if distensibility of an individual vessel was diameter dependent, for example higher distensibility seen only in larger vessels, when cardiac output increased, flow would preferentially be directed to larger vessels over smaller ones at a bifurcation of two vessels of different diameters, and flow (mal)redistribution would result (94). If this diameter-independent characteristic of vascular distensibility is also true of the pulmonary arteries and veins of the horse, it could explain why the distribution of pulmonary blood flow does not change a great deal from rest to exercise in horses (15).

To the best of my knowledge, *regional* differences in pulmonary vessel mechanical properties have not been reported in any species to date, and without such a precedent, it is difficult to predict that they occur in the horse. However, *should* regional heterogeneity in equine small pulmonary vessel distensibility exist, resulting differences in resistances to flow in vessels supplying and/or draining pulmonary capillaries would impact regional pulmonary capillary pressures, and as a result, perhaps predict regional propensity for EIPH and associated pathology.

Increases in collagen content of a vessel are associated with altered mechanical properties – specifically increased stiffness (12, 30). Increased collagen content of remodeled equine pulmonary veins has already been reported (38), and this change occurs in caudodorsal but not in cranioventral lung regions (231, 233). These reports describe remodeling changes in horses with advanced/severe EIPH, and reports of EIPH-associated pathology in racehorses that lack a clinical history of severe EIPH do not exist at this time.

Based on the observations in the 2013 pathology mapping paper that demonstrate that the full constellation of EIPH lesions (hemosiderin and fibrosis) cannot occur without colocalized venous remodeling, but that remodeling can occur on its own (233), it is proposed that remodeling is an early change in EIPH pathogenesis. It is reasonable to suggest therefore that alterations in venous wall stiffness due to remodeling processes will be detectable in horses that are training and racing, but do not necessarily have severe EIPH pathology. In the event that vein walls in caudodorsal lung are stiffer in horses that train and race compared to cranioventral veins, and compared to veins from horses that do not race, this information will help further corroborate the pulmonary vein as a key component of early EIPH pathogenesis. Increased stiffness resulting in diminished venous compliance in caudodorsal lung only will inhibit pressure-mediated venous dilation, and cause increased resistance to blood flow in those veins. This in turn will increase pulmonary capillary pressure in caudodorsal lung, and increase the propensity for capillary rupture and EIPH in this lung region.

In summary, investigation of the mechanical properties of small pulmonary vessels of the horse have not heretofore been reported, and, based on the outlined rationale –

alterations in these properties of pulmonary veins could be implicated in both development and progression of EIPH.

The study outlined in Chapter 3 of this dissertation, was designed to test the following hypotheses:

Mechanical properties of small pulmonary arteries and veins do not differ by region in control, unraced horses. Also, pulmonary veins, but not pulmonary arteries from horses with a recent racing history, have increased wall stiffness compared with veins from horses that have never raced, and this change is limited to veins from caudodorsal lung only.

Regulation of pulmonary vascular tone

In general, the pulmonary circulation can be described as a high-flow, low pressure system, that functions to match perfusion to ventilation. This is achieved by both passive and active factors that affect pulmonary vasculature. Vascular wall mechanical characteristics that are investigated in the first study of this dissertation are among the passive factors that exert control over pulmonary vascular resistance (11). However, active control of vascular tone must also be considered an important variable in determining pulmonary vascular resistance (PVR). Tone is determined by the state of contraction of vascular smooth muscle, which affects vessel diameter, and resistance to flow in that vessel segment as a result. Small changes in vessel diameter exert a large effect on resistance, as defined by the Hagen-Poiseuille relationship. Of particular significance to EIPH pathogenesis, regulation of resistance to flow in small pulmonary arteries and veins directly impacts pulmonary capillary pressure. Active regulation of the pulmonary circulation is achieved by means of a complex combination of neural and humoral influences (11).

Autonomic control of pulmonary vascular tone

Neural control of the pulmonary circulation is provided by the autonomic nervous system which directly innervates the pulmonary circulation, and also exerts its effects through release of circulating (humoral) vasoactive factors (34).

Sympathetic nervous system activity is mediated by α and β-adrenergic receptors (11). In general, activation of α-adrenoreceptors causes vasoconstriction of pulmonary arteries (83), although activation of α_2 receptors on pulmonary artery endothelium cause relaxation of porcine pulmonary arteries (165). Both β_1 and β_2 receptors have been identified on pulmonary vessels (154), although β_2 receptors likely predominate (83), and their activation results in vasodilation (133).

The extent of sympathetic innervation of the pulmonary vasculature varies significantly between species, and this was reviewed in detail by Barnes and Liu in 1995 (11), however to the best of my knowledge, whether equine pulmonary vessels (arteries and veins) of the caliber evaluated in these studies are innervated with postganglionic sympathetic fibers has not been reported.

Muscarinic receptors, when bound by acetylcholine, mediate parasympathetic control of vascular tone, and 4 of the 5 subtypes (M_1 – M_4) have been identified in pulmonary vessels (11). Acetylcholine (Ach) is reported to cause both vasoconstriction and vasodilation of pulmonary arteries (133). Norel *et al* reported that both the subtype of muscarinic receptor, and whether the receptor was located on the vascular smooth muscle (for contraction) or on the endothelium (for dilation) were factors in determining a vessel's response to Ach (148).

As is the case with sympathetic innervation, there are significant differences between species in the extent of parasympathetic innervation of the pulmonary vasculature (11), and whether small caliber equine pulmonary arteries and veins possess parasympathetic nerves is not reported to date.

Numerous investigators have evaluated the effect of the sympathetic and the parasympathetic branches of the autonomic nervous system on the pulmonary vasculature as a whole circuit (rather than individual vessels), and in essence, unopposed sympathetic stimulation is reported to increase pulmonary vascular resistance (91), whereas vagal stimulation (in the face of adrenoreceptor blockade) results in dilation of the pulmonary vascular bed (143). However, both branches of the autonomic nervous system work in concert *in vivo*. Based on experiments performed in conscious dogs in which complete autonomic ganglion blockade, and specific cholinergic and adrenergic receptor blockers were used, the net effect of autonomic nervous system activity on the pulmonary circulation at rest is mild vasodilation, which is predominantly mediated by sympathetic β-adrenergic activity (140).

In the exercising horse, as is the case in other mammals, sympathetic activity is increased, while parasympathetic outflow correspondingly decreases (133). During exercise the horse's cardiac output increases (44), circulating catecholamine levels increase approximately 10-fold (191) and pulmonary vascular resistance (PVR) decreases (128).

Although recruitment and passive distension of vessels certainly contributes to reduced PVR, whether the sympathetic nervous system could play a role in the PVR decrease has been investigated. In sheep, during exercise, both α- and β-receptor activation occurs, however the net effect on pulmonary vascular resistance is neutral i.e. PVR during

exercise in the face of α- and β-receptor blockade was not different to PVR during control runs (93). Experiments performed in exercising swine indicated that β-adrenoreceptor-mediated vasodilation was appreciable, that there was minimal impact of α-receptor activation, and some vasodilation occurred secondary to muscarinic receptor activation (203).

Regional control of pulmonary vascular tone

That the autonomic nervous system plays a role in exerting changes in pulmonary circulation resistance during exercise is clear, however my interest in the autonomic control of vessel tone is less focused on overall PVR in the pulmonary circulation, but rather on regional control of pulmonary capillary pressure. As already discussed, resistance to flow in the pulmonary arteries and veins supplying and draining the pulmonary capillaries determine capillary pressures, and regional differences in the active control of small pulmonary vessel tone could explain why EIPH lesions have a distinct predilection for caudodorsal lung (233).

An existing precedent for regional heterogeneity of small pulmonary vessel reactivity in any species does not exist in the literature at present, however regional differences in reactivity of large pulmonary arteries have already been reported in both the pig (176), and in the horse (164). In the pig, greater relaxation to acetylcholine-induced nitric oxide release was observed in large (3.6 mm O.D.) dorsal arteries, compared to ventral (176). Interestingly, and also in pigs – experimental endotoxemia is reported to increase perfusion to dorsal lung, and reduce that of ventral lung, which is also suggestive of regional differences in vascular responses of large pulmonary vessels to a vasoactive

stimulus (51). In the case of large (6 mm O.D.) equine pulmonary arteries, vessels from caudodorsal lung relaxed in response to methacholine (a muscarinic receptor agonist) in an endothelium-dependent manner, whereas those vessels in cranioventral lung contracted after a transient relaxation, again, in an endothelium dependent manner (164). When applied to the whole lung, these data provide a possible explanation (secondary to anatomy) for the heterogeneous distribution (caudodorsal predilection) of pulmonary blood flow in the horse lung (15, 76).

The second study of this dissertation was designed to test the following hypothesis:

Regional differences in patterns of vascular reactivity to adrenergic and cholinergic agonists in small pulmonary arteries and veins exist, and do so in a manner that will predict the predilection of EIPH pathology for the caudodorsal lung region.

Phenylephrine and isoproterenol were selected as the alpha- and beta-adrenergic agonists respectively, and the muscarinic agonist methacholine was chosen to evaluate muscarinic responses.

As discussed above, whether the vessels that are studied possess sympathetic and parasympathetic nerve fibers remains ambiguous at this time. In general terms however, any response to an agonist will be interpreted as indication that the receptor in question is present in the vessel wall, and therefore susceptible to binding by agonists *in vivo,* whether the source of that agonist is from an adjacent nerve terminal, or the circulating blood.

U 46619

In order to evaluate isoproterenol and methacholine, both of which are expected to relax pulmonary vessels, pre-contraction of vessels to provide tone is necessary to evaluate

drug-induced relaxation. The thromboxane A_2 analog U 46619 is a reliable vasoconstrictor in many vessel types, and was selected for this purpose. Thromboxane A_2, an arachidonic acid metabolite derived from endothelial cells and platelets (147, 168) is a pulmonary vasoconstrictor that is implicated in endotoxemia-associated pulmonary hypertension (103) and other pulmonary diseases. U 46619 mediates its effects by binding to $G_{12,13}$ coupled TP (thromboxane) receptors on vascular smooth muscle cells, which results in enhanced calcium sensitivity of myofilaments, and some increases in intracellular calcium concentration and (40, 194). Enhanced sensitivity to U 46619 in pulmonary veins compared to pulmonary arteries has been reported in multiple species, including sheep, dogs and guinea pigs (10, 99, 187). Ovine pulmonary veins that are less than 1 mm in diameter produce significantly more thromboxane A_2 than both larger veins, and pulmonary arteries (84). A stable analogue of thromboxane A_2 has been demonstrated to increase pulmonary capillary pressure by selective venoconstriction (189). Based on information from other species, any significant *in vivo* activity of thromboxane A_2 in the horse most likely involves pulmonary veins. The role of thromboxane in regulation of vascular tone during exercise however, is thought to be minimal (144), and therefore thromboxane A_2 is not likely to be a contributing factor to the propensity of caudodorsal lung to develop EIPH lesions.

Phenylephrine

Binding of phenylephrine to the G_q protein coupled α_1 adrenoreceptor on vascular smooth muscle cells activates phospholipase C, which in turn mediates a signaling cascade that

both causes release of calcium from sarcoplasmic reticulum and inhibition of myosin light chain phosphatase, and ultimately – smooth muscle contraction (194).

Phenylephrine is a reliable pulmonary arterial constricting agent (83, 165), and has already been demonstrated to exert vasoconstrictor effects on large (1.5 – 4 mm in diameter) equine pulmonary arteries (117), and veins (63).

Isoproterenol

When bound to either β_1 or β_2 adrenoreceptors on vascular smooth muscle cells, isoproterenol, by means of G_s protein coupling increases adenylate cyclase activity and production of the second messenger cAMP. cAMP in turn decreases myosin light chain kinase activity (*via* protein kinase A) and smooth muscle relaxation results (182). More recently, the role of endothelial β adrenoreceptors in vasodilation is being investigated, and increased nitric oxide synthesis/release as the proposed mechanism (216, 228).

Isoproterenol is reported to cause relaxation in both pulmonary arteries (154) and in pulmonary veins, although, the response of rat pulmonary veins to isoproterenol was affected both by the caliber of vessel and the tension (corresponding to pressure) at which vessels were normalized before the experiment (19).

Methacholine

Methacholine is a non-selective muscarinic receptor agonist that is commonly used to mimic acetylcholine in an experimental setting. Muscarinic-receptor mediated vasodilation is typically mediated by release of endothelium-derived relaxing factors (EDRFs) such as the prototypical relaxant nitric oxide (NO) and/or prostanoids, commonly prostacyclin

(PGI_2); on the other hand, vasoconstriction can result from production of vasoconstrictor prostanoids also known as endothelial derived constricting factors (EDCFs)(236), and/or direct binding of vascular smooth muscle muscarinic receptors (133). Inhibition of nitric oxide synthase and/or cyclooxygenase are commonly used techniques employed to investigate the relative roles of nitric oxide and arachidonic acid metabolites respectively in muscarinic-receptor mediated vasomotion (40, 164).

Muscarinic-receptor binding can cause vasodilation in precontracted pulmonary arteries and veins (149), and again – heterogeneity of the magnitude of responses in vessels of different diameters is reported in rats, pigs and sheep (99, 108, 239). On the other hand, acetylcholine is also reported to cause contraction in both pulmonary arteries (horse) (164) and veins (208). As already mentioned, experiments by Norel *et al* support the concept that whether muscarinic activation results in constriction or relaxation of a blood vessel is determined by the relative distribution of muscarinic receptors (or various subtypes) on vascular smooth muscle and endothelium (148).

Furosemide

Furosemide is the only pharmacotherapeutic used in the treatment of EIPH that has demonstrable (albeit partial) efficacy (74). Although its effects are most often attributed to sustained attenuation of pulmonary capillary pressures (54, 123) associated with decreased plasma volume (72), the drug's pulmonary venodilator properties (59) are also of interest but as of yet, unstudied in the horse.

That the distribution of pulmonary blood flow in the horse both at rest and at exercise is altered by furosemide, while cardiac output is not, strongly suggests that

furosemide has a direct vasoactive effect on the equine pulmonary vasculature (43). Indeed, besides pulmonary vasoactive properties of the drug, few other, plausible explanations for the observation that furosemide can alter pulmonary blood flow distribution exist.

If vasoactivity contributes to the efficacy of the drug in EIPH, then to have a protective effect on pulmonary capillary pressures, furosemide should dilate pulmonary veins, and not arteries. This selectivity for pulmonary veins and not arteries has been demonstrated in the dog (59).

For these reasons the following hypothesis was also investigated in the second study of this dissertation: *In the small pulmonary veins of the horse, furosemide dilates pulmonary veins but not pulmonary arteries independent of lung region.*

There does not appear to be consensus in the literature regarding the mechanism of action of furosemide as a venodilator. Pickkers *et al* report that furosemide effectively relaxed pre-contracted dorsal hand veins, but that the effect was completely abolished by pretreatment with indomethacin, which implicates local prostanoid synthesis as a key mechanism (170). Prostanoid involvement of furosemide's activity as a vasodilator is also supported by data from isolated dog lung lobe preparations (116). Others propose, and have data to support a direct effect of furosemide on vascular smooth muscle, by inhibition of chloride-dependent Na^+/K^+ co-transport resulting in reduced intracellular Na^+, the replacement of which drives Ca^{2+} out of the cell (59).

It is worth noting however that the study of furosemide activity on canine pulmonary veins encompassed drug concentrations between 33 and 1000 times as high as the plasma level of furosemide in horses 1 hour after administration of 1 mg / kg, which is

an effective dose in EIPH treatment (24, 59). Therefore vasoactivity would probably only occur in the event of accumulation of active drug in lung tissue. Significant accumulation of furosemide in lung tissue is not considered likely, as furosemide is highly protein bound in plasma, and excreted before significant metabolism. Indeed, in dogs, at 1 minute after maximal diuresis post-furosemide administration, the ratio of unaltered furosemide in lung tissue to that in plasma is 0.26, indicating that little to no accumulation of drug occurs in the dog lung (31). While the pharmacokinetics of furosemide in the horse are documented (24), to the best of my knowledge, equine tissue accumulation studies have not been reported.

Summary

Little is known about the mechanisms of control of vascular tone in the small pulmonary arteries and veins of the horse. As these vessels affect pulmonary capillary pressures, knowledge of regional differences in vessel reactivity, in particular under the high sympathetic/low parasympathetic outflow conditions of exercise could contribute further understanding to the pathogenesis of EIPH.

Section 3: Pulmonary Venous Remodeling

This section is designed to provide further information on vascular remodeling, focusing specifically on the role of venous remodeling in EIPH. This will provide both context and a rationale for the study entitled "Effects of exercise on markers of venous remodeling in lungs of horses."

Venous remodeling in EIPH

Remodeling of small pulmonary veins as a component of EIPH pathology was first reported in 2008 (231). Horses that were retired from racing due to severe EIPH were studied, and in caudodorsal lung, remodeling of intralobular, septal and sub-pleural veins is detailed along with other classic features of EIPH pathology such as hemosiderin accumulation, angiogenesis and interstitial fibrosis (152, 157). The predominant feature of remodeled vessels is expansion of the adventitial compartment with what is described as a "prominent collar of mature collagen" (231). Collagen deposition extends to the media/intima (i.e. between the external elastic lamina and vessel lumen) in some vessels, causing luminal obstruction. This reduction in lumen area was further defined in a subsequent study that used morphometric techniques to confirm that the luminal perimeter of veins in caudodorsal lung regions with the most severe histopathology was significantly less than in other, less severely affected regions (38).

The results of an extensive study of the pathology of horses that raced in the US and were retired due to severe EIPH demonstrated conclusively that EIPH pathology, including venous remodeling, was most common and most severe in caudodorsal lung (233). Hemosiderin and remodeling were either found together, or mild remodeling was found on

its own without hemosiderin. Furthermore, interstitial fibrosis almost always (in 99.7 % of samples) occurred with colocalized venous remodeling. These data strongly suggest that remodeling is both pivotal in the disease process, and is an earlier pathologic event than fibrosis.

Vascular remodeling

In contrast to acute changes in vessel tone such as those induced by autonomic agonists, vascular remodeling instead describes enduring changes in vessel wall structure that generally occur in response to hemodynamic stimuli (67).

Remodeling responses in veins from the systemic circulation have been studied extensively as a result of the common practice of vein grafting in coronary artery bypass procedures, and subsequent issues with maintenance of long-term vein graft patency due to remodeling (240) . When systemic veins are exposed to arterial conditions of flow (resulting in shear stress) and increased transmural pressure (resulting in circumferential stress) in an experimental setting, increased wall thickness is observed as early as one to two weeks (28, 64). Venous remodeling and resultant stenosis in the context of grafting is generally characterized by significant neointima formation, a process that is modulated by both shear stress and pressure (14, 61).

In contrast to systemic veins, remodeling in pulmonary veins is dramatically understudied. Extensive investigations into remodeling of pulmonary arteries exist however, and are performed mainly in the context of pulmonary hypertension in humans. While there must be some common ground between pulmonary arterial and venous remodeling in terms of surrounding environment and remodeling stimuli, I propose that

pulmonary artery remodeling is not necessarily an ideal template for the same process in pulmonary veins for the following reasons. Temporally, embryonic pulmonary vein development long precedes that of pulmonary arteries (37) and recent molecular investigations have further defined arterial-venous heterogeneity (3). Data from Chapters 2 and 3 of this dissertation further support the contention that pulmonary veins and arteries are clearly distinct from one another in both their anatomy and physiology (196).

Pulmonary venous remodeling - etiopathogenesis

To the best of my knowledge, and with the exception Chapter 1 of this dissertation, there are no reports of pulmonary venous remodeling that is associated with exercise in any other species (196). There are however accounts of such remodeling responses to hemodynamic stimuli similar to those experienced by the exercising horse – namely increased pulmonary blood flow (43) and intravascular pulmonary venous pressures (88, 124).

In general, two types of hemodynamic stresses are applied to vessel walls by the blood within the lumen; namely shear stress caused by blood flow and circumferential or hoop stress caused by pulse pressure (159). Circumferential stress is applied to all components of the vessel wall, whereas shear stresses mostly affect the endothelium. While alterations in shear stress are widely recognized as a cause of vascular remodeling (217) it is difficult to estimate whether this is a significant stimulus in EIPH-associated venous remodeling, in large part because very little is known about *in vivo* flow patterns (and resulting shear stresses) in the small pulmonary veins of any species. Remodeling associated with increased shear stress typically results in "outward remodeling" which

thickens vessel walls, specifically the smooth muscle of the *tunica media* (21, 215), but ultimately results in vessel diameter expansion to promote return of shear stress to normal levels (159). In contrast, a reduction in venous diameter reduction is reported in EIPH-associated venous remodeling (38). Additionally, all EIPH-remodeled vessels, even those that are mildly affected, demonstrate adventitial compartment expansion by collagen, in most cases without expansion of the smooth muscle of the *tunica media* (231, 233). I propose therefore that increased shear stress in response to increased pulmonary blood flow during exercise is not the predominant stimulus driving development of pulmonary venous remodeling. The rest of this discussion will be focus mainly on increased intravascular pressure (and resulting circumferential wall stress) as a stimulus for remodeling.

Experimental data exist that demonstrate a remodeling response of pulmonary veins to increased venous pressure. When sheep were exposed to a continuous pulmonary arterial air embolus, a technique that increased total pulmonary resistance and pulmonary vascular pressures, venous wall thickening was observed within 4 days of exposure (86). Thickening of the adventitia was noted in some vessels by day 12.

In left heart failure pathologic vascular remodeling is usually seen in the pulmonary veins before arterial changes occur, due to increased "retrograde" pressure in the pulmonary circulation (82, 213). Mitral stenosis for example, results in significant thickening of small pulmonary vein walls due to fibrosis of the intima and adventitia (25) in response to persistent venous hypertension.

Pulmonary venous occlusive disease (PVOD) is a rare form of pulmonary hypertension that is associated with high mortality (81). PVOD is characterized by fibrotic

intimal expansion of small pulmonary veins and venules, and in some patients alveolar hemorrhage and hemosiderosis (78). PVOD is frequently idiopathic, and the initiating trigger for disease development remains undetermined for many patients (81). However, sustained venous hypertension is thought to be a significant stimulus for ongoing venous remodeling in PVOD, as the vascular changes observed resemble those described in mitral stenosis and other causes of pulmonary venous hypertension.

Role of venous remodeling in EIPH

It is particularly interesting that pulmonary arteries in EIPH-affected lungs are not routinely remodeled, while the small veins are significantly affected (231). Cardiac output and pulmonary artery pressures are dramatically increased in the exercising horse (44, 124), yet only venous pathology is observed. Conditions in humans in which venous pathology either occurs exclusively, or to a greater degree than arterial remodeling include left heart failure, and PVOD, and remodeling in both is attributed in some degree to venous hypertension specifically (25).

A galloping horse has a maximum heart rate of approximately 220 b.p.m. and a stroke volume of approximately 1.5 L (44). Left heart diastole is estimated therefore at 0.14 seconds, and minute volume is 330 L/minute. In the Operation Everest II studies performed on elite human athletes in the early 1990s, investigators report a "remarkably" close relationship of right atrial and pulmonary artery wedge pressures (P_{wp}) during exercise, although it is noted that a 1 mmHg rise in right atrial pressure resulted in a higher (1.4 mmHg) rise in P_{wp} (173) which further loads the venous compartment. Supplying the left ventricle with 1.5L of blood within 0.14 s requires extremely high filling pressures,

which is provided by the right heart and pulmonary arterial driving pressure, however maintenance of this filling pressure during exercise also results in necessary, but sustained "retrograde" pressure increases in the pulmonary venous system specifically. I also consider it plausible, that the equine pulmonary arterial system positioned as it is, immediately downstream from the right ventricle, is inherently better adapted to manage a hypertensive stimulus without pathologic consequences than the venous circulation, and if this were the case, it could go someway toward explaining the "selective" remodeling observed in pulmonary veins in EIPH.

Whether arteriovenous shunting occurs during exercise such as is reported in other species, including dogs (202) has been preliminarily investigated in the horse, and was not demonstrable through the use of microsphere injection (125). Use of another sensitive technique, contrast echocardiography has demonstrated shunting during exercise in human subjects (114), and if this phenomenon occurs in exercising horses, it may result in further volume loading of the pulmonary venous circulation, and further compound pulmonary venous hypertension.

Although it is possible that venous remodeling is a *result* of EIPH, to the best of my knowledge no other such conditions (i.e. venous remodeling in response to local hemorrhage) are reported in the literature. Therefore I propose that venous remodeling assumes a key role early in EIPH pathogenesis, as follows:

Exercise-associated elevations in cardiac output result in dramatically elevated pulmonary artery pressures in the galloping horse. These high pressures are transmitted to the venous circulation in order to provide elevated left atrial filling pressures. Furthermore, maintenance of high cardiac output from the left ventricle during exercise

contributes to sustained retrograde elevations in left atrial, and therefore pulmonary venous pressure during exercise. It is widely accepted that left atrial pressures are transmitted upstream without dampening, in a fully-recruited pulmonary circulation (141) With repeated training, these periods of venous hypertension result in venous wall remodeling, specifically in the caudodorsal lung, which is the region in which blood flow and presumably, therefore intravascular pressures are highest (15). Pulmonary capillary breaking strength is exceeded in some capillaries during exercise, and hemorrhage results. Those capillaries that are drained by remodeled veins are even more susceptible to rupture during exercise as venous wall compliance is reduced and in some cases, venous luminal area is diminished. Those capillaries are exposed to pressures approaching arterial values and rupture. With each exercise bout the injurious cycle is repeated and compounded, ultimately resulting in clinically detectable hemorrhage, significant pulmonary pathology, and potentially impaired performance.

To validate this pathogenesis hypothesis, it is necessary to implicate venous remodeling as an early, pivotal event in EIPH (rather than merely a result of local hemorrhage). Therefore I designed a study investigate whether intense exercise could be associated with venous remodeling *before* the development of severe EIPH pathology. The following hypothesis was investigated in Chapter 4 of this dissertation:
Two weeks of intense exercise will alter mRNA and protein expression of those factors known to mediate vascular remodeling in the pulmonary veins from caudodorsal but not cranioventral regions of lungs of horses in a manner that favors venous remodeling.

Mechanisms of venous remodeling

mRNA and protein production precede histopathologic change. Therefore, in order to evaluate remodeling as early in its development as possible, mRNA and protein levels of factors known to mediate vascular remodeling were studied after just two weeks of intermittent intense exercise.

Achieving a balance between detecting the earliest effects of exercise in pulmonary vein walls, while providing a stimulus that was adequate to invoke such change was to prove challenging. Reports from other species (sheep and rabbits) describe structural alterations in remodeling veins as early as 4 (86) to 7 (64) days after initiation of a continuous hypertensive stimulus. With a goal of detecting pre-structural changes, six episodes of an intermittent hypertensive stimulus over a 14-day period were deemed sufficient.

As venous remodeling in EIPH follows a distinctive regional pattern, and veins from cranioventral lung are unaffected (233), expression of mRNA and protein of the following list of factors were studied in veins from 2 distinct regions of lung – caudodorsal, and cranioventral: Collagen type I, tenascin-C, matrix metalloproteinases (MMP) MMP-1 and MMP-9, tissue inhibitors of metalloproteinases (TIMP) TIMP-1 and TIMP-2, endothelin-1 (ET-1), platelet-derived growth factor (PDGF), transforming growth factor beta (TGF- β) and vascular endothelial growth factor (VEGF).

Until recently the role of *tunica adventiti*a in vascular remodeling has been somewhat overlooked in favor of the *tunica intima* and *tunica media* and their predominant cell types, the endothelial and smooth muscle cell respectively. In the field of pulmonary hypertension research there is mounting evidence that the adventitia and the cells contained therein act as a key injury-sensing and regulatory center for pulmonary arteries

(199, 200). In remodeled veins in EIPH-affected lung, the *tunica adventitia* of these vessels is the most commonly, and most severely affected compartment (231, 233). For this reason, I consider it likely that the adventitia of pulmonary veins also plays a significant role in mediating the remodeling response in EIPH.

The most common cell type in the *tunica adventitia* is the adventitial fibroblast. When activated, the adventitial fibroblast rapidly differentiates into a myofibroblast, and fulfills multiple functions, some of which include production of extracellular matrix proteins including collagen and tenascin-C, and a variety of growth factors (201).

The first description of remodeled veins in EIPH details marked collagen deposition in the adventitia of remodeled vessels (231). This observation was supported statistically when morphometric analysis demonstrated increased collagen content in vessels from affected lung regions (38). Collagen is commonly increased in the extracellular matrix of remodeled veins from other species (humans and rabbits) (25, 235). For these reasons collagen mRNA and protein expression were evaluated in this study.

Tenascin-C is another important extracellular matrix protein that is expressed by remodeling tissues during fetal development and in disease states. For example, tenascin-C expression is upregulated in remodeling veins in both humans (222) and rodents (2). The adventitial fibroblast is a major source of tenascin-C in injured vessels (223), therefore the expression of tenascin-C mRNA and protein were selected for evaluation in this study.

The proteolytic matrix metalloproteinases MMP-2 and MMP-9 are sub-classified as gelatinases, and their substrates include extracellular matrix (ECM) components such as collagen and elastin (218). TIMPS are endogenous inhibitors of MMP activity, and bind MMPs in a 1:1 stoichiometry (171). Although 4 TIMPS have been characterized in

vertebrates, TIMP-1 binds preferentially with MMP-9, and TIMP-2 with MMP-2 (26), therefore TIMP-1 and TIMP-2 were chosen as candidates for study. MMPs and TIMPs act in concert to modulate the turnover of extracellular matrix of vessel walls in both health and disease. For example, pressure-associated vascular remodeling is generally associated with increases in MMP-2 and MMP-9 expression (27, 29) and while TIMP expression can be either unaltered or decreased (235) in remodeled vessels, the overall MMP:TIMP ratio is frequently increased (23, 90). Most vascular wall cell types, including adventitial fibroblasts, produce MMPs (26). It has been demonstrated that increased MMP and/or decreased TIMP expression is necessary for the migration of activated fibroblasts from the adventitia to the media and intima (188) thereby allowing extension of the remodeling process to the rest of vessel wall. Inward remodeling such as this is observed in the more severely remodeled veins of EIPH-affected equine lung (233).

Endothelin-1 (ET-1) is reported to be a potent vasoconstrictor of both pulmonary and systemic vessels in the horse, and this effect is predominantly mediated through the ET_A receptor (13). In other species however, endothelin has been implicated in vascular remodeling processes as a mitogenic factor for smooth muscle (35) and as a comitogen that augments TGF-β-induced collagen I production (105). Increased ET-1 expression is reported in a hypoxic model of pulmonary venous remodeling (204), and administration of an ET_A receptor antagonist is reported to inhibit pressure-mediated remodeling in porcine veins (224). Although vascular smooth muscle is an important target tissue in both acute and chronic responses to endothelin, endothelial cells are the predominant source of ET-1, the majority of which is secreted at the basolateral cell surface (96).

Platelet-derived growth factor (PDGF) is a mitogenic factor that is produced by and acts upon many cell types, including vascular smooth muscle cells, and fibroblasts (65). PDGF is over-expressed in small remodeled pulmonary arteries in patients with idiopathic pulmonary arterial hypertension compared to arteries from normal subjects (166). Upregulation of PDGF mRNA in porcine veins that are exposed to arterial conditions of pressure and flow is also reported (48). The role of PDGF in vascular remodeling processes is further substantiated by experimental and clinical reports of effective reversal of pulmonary arterial remodeling by treatment with the tyrosine kinase inhibitor imatinib, a PDGF receptor antagonist (53, 181).

Transforming growth factor beta (TGF-β) is a pleiotropic cytokine, and the prototypical member of a large group of cytokines whose functions span development, health and disease in many tissues (58). The role of TGF-β in pulmonary artery remodeling has been studied extensively, as mutations of the TGF-β receptor BMPR2 are strongly associated with familial pulmonary arterial hypertension (118). The role of TGF-β in pulmonary venous remodeling remains undefined, however it is implicated in remodeling of systemic veins. Vein grafts that have undergone multiple stenoses express more TGF-β than veins that have never remodeled or have only stenosed once (146), and treatment with TGF-β antisense mRNA prevents collagen deposition in femoral vein grafts in rats (234). Furthermore, TGF-β is recognized to play a key role in the differentiation of fibroblasts to activated myofibroblasts (120).

The final growth factor that was selected for evaluation in this study is vascular endothelial growth factor (VEGF). VEGF is highly expressed in the lung (219), and its production by endothelial cells can be induced by many growth factors including TGF-β

(167). In pulmonary arterial hypertension VEGF protein has been demonstrated in plexiform, lumen-obliterating endothelial lesions (214). Its role in vascular remodeling is supported by the observation that VEGF blockade prevents neointima formation in arteries (241).

Summary

In summary, to implicate pulmonary venous remodeling as a pivotal and early change in EIPH pathogenesis, a study was designed to evaluate the early expression patterns of extracellular matrix proteins, their modulating enzymes, and various growth factors in pulmonary veins from 2 lung regions of horses after two weeks of a transient hypertensive stimulus (exercise).

Section 4: Experimental technique development

This section provides supplemental information on why wire myography was chosen as a key experimental technique, and the rationale and theory behind vessel normalization strategy.

Wire myography

Before wire myography was determined to be the most suitable technique to evaluate both the mechanical and pharmacological properties of small caliber equine pulmonary vessels, attempts were made to utilize pressure myography. In brief, pressure myography involves mounting each end of and securing the vessel of interest on two patent glass canulae that are themselves attached to a sealed, and pressurized system. The vessel walls and lumen can be monitored continually, and responses of vascular smooth muscle, and the resulting alterations in vessel diameter recorded in real time. Some advantages of pressure myography over wire myography include equal and constant distribution of intraluminal pressure conditions to the vascular wall, which better-emulates *in vivo* conditions, and also, intravascular pressure can be easily and rapidly adjusted by means of a fluid column that communicates with the canulae-vessel system.

When equine pulmonary arteries between 100 and 400 μm O.D. were mounted on the pressure myograph however **(Figure 1)**, it was difficult to maintain pressure in any vessel segment despite careful dissection technique. The reason for this is attributed to the frequent, and asymmetrical branching pattern of the pulmonary vasculature. For example, supernumerary arteries branching from a parent artery, usually at 90 degrees, can be significantly smaller in diameter that the vessel from which they originate (210). Due to an

unacceptable failure rate using this technique, the decision was made to switch to conventional wire myography.

Mulvaney and Halpern developed wire myography in the 1970s, and they first reported its use as a tool to investigate lengths of small arterial resistance vessels obtained from rat mesentery (138, 139). Wire myography is now a commonly used technique for the study of small blood vessel characteristics.

The wire myograph chamber is filled with physiologic buffer solution, which can be warmed, and perfused with gas(es), and to which pharmacologic agents may be added. Small vessels (< 400 μm O.D.) are mounted as a cylinder on two parallel 40 μm O.D. stainless steel wires in the chamber. One wire is attached at two points to a metal jaw that is controlled by a micrometer screw, and the second wire attached to an identical jaw that is attached in turn to a sensitive force transducer. **(Figure 2)**. In this arrangement, the parallel wires can be distracted from one another by use of the micrometer, thus controlling vessel internal circumference (L) and enabling evaluation of the tension in the vessel wall (T) evaluated as various stimuli are applied.

The formula used to calculate internal circumference (L) is

$$L = (\pi + 2)d + 2f$$

where *d* is wire diameter, and *f* is the distance between the wires. The wire circumference (π d) accounts for the 2 outer halves of the wire that are in contact with the vessel, but it does not account for half of wire diameter (*d/2*) that is closest to the center of the vessel, and that contributes to the total diameter, even when the wires are touching (or $f = 0$). For one "side" side of the vessel, $2 \times d/2 = d$. For both "sides" of the vessel *d* is doubled. **(Figure 3)**.

Assuming a thin-walled tube condition (which is the case in small caliber vessels such as those studied) the Laplace relationship can be applied, and the equivalent transmural pressure (P) calculated from wall tension (T)(which is read by the pressure transducer) and vessel circumference (L)(as determined by the micrometer screw).

$$P = T/(L/2\pi)$$

A degree of preexisting tension is necessary in order to align smooth muscle fibers for optimal development of force when stimulated (7). Before experiments, vessels are normalized to similar conditions of wall tension that permit comparisons to be made between vessels of different diameter.

Many investigators elect to set a value for L such that the passive resting tension on vessel walls is equivalent to a physiologically relevant transmural pressure, for example 100 mmHg in systemic arteries.

In the pharmacology experiments outlined in Chapter 3 of this dissertation, a different approach was taken for the following reason - maintenance of the very minimal tension required to emulate physiologic pulmonary venous transmural pressure (approximately 10 – 15 mmHg) throughout an entire experiment proved challenging, which led me to become concerned that not all vessels were being exposed to similar conditions at all times. Normalization was therefore performed with the goal of determining each vessel's optimal T for subsequent force generation. Vessels were tested with 60 mM K^+ solution over a range of passive wall tensions. The tension at which the maximal response to KCl was generated was determined to be that vessel's optimal passive tension, and the tension at which all subsequent experiments were performed.

APPENDIX

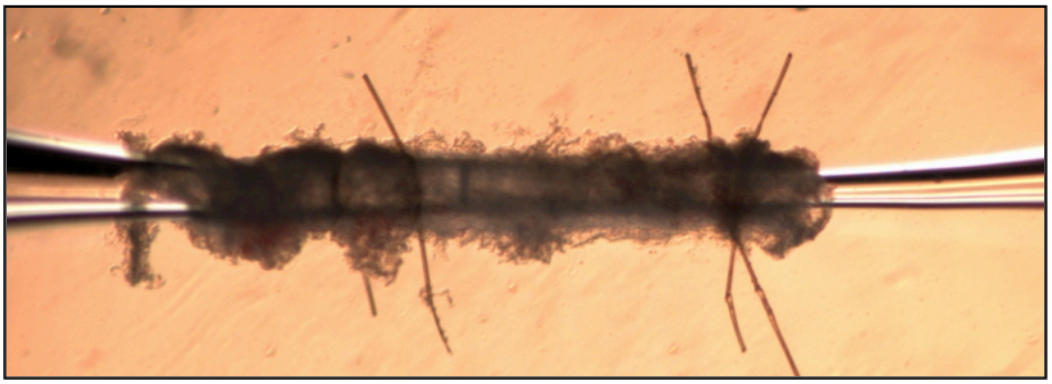

Figure 1 Equine pulmonary artery on a pressure myograph. The artery is secured to 2 glass canulae by suture material.

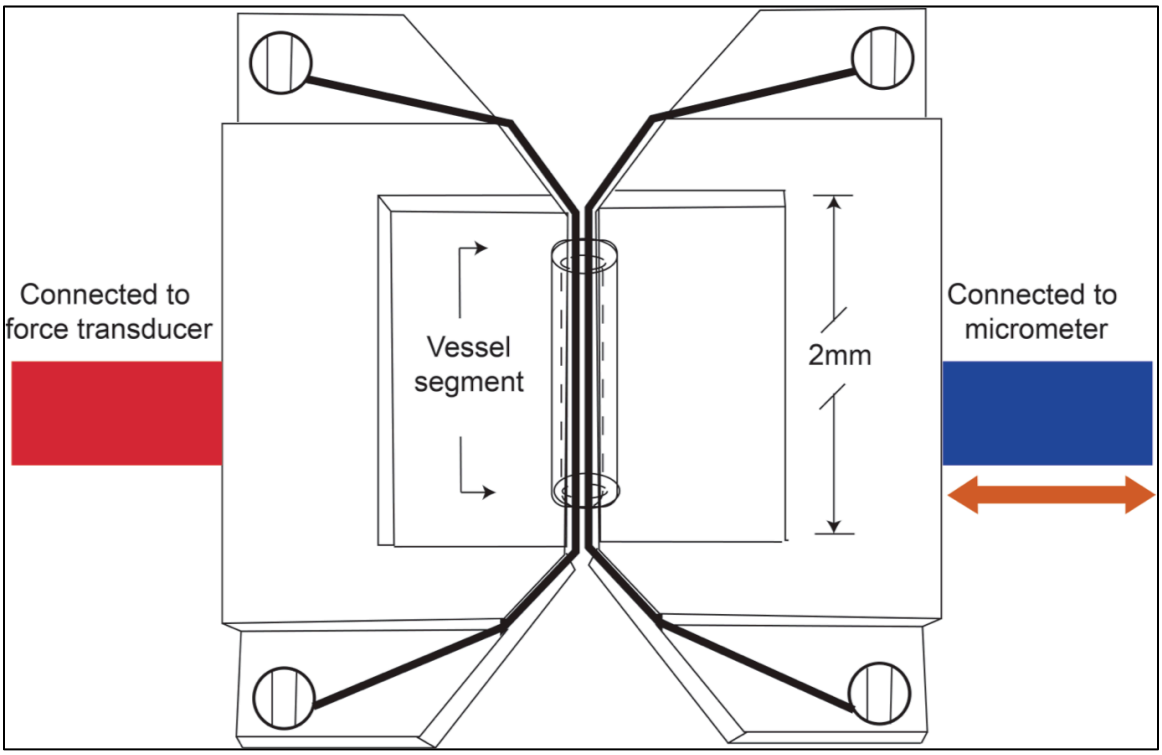

Figure 2 Schematic of a vessel mounted on wire myograph. Vessel is mounted as a cylinder on two stainless steel wires. Each wire is attached to a metal jaw, one of which can be moved by a micrometer screw (right of image) and the other is connected to a sensitive force transducer.

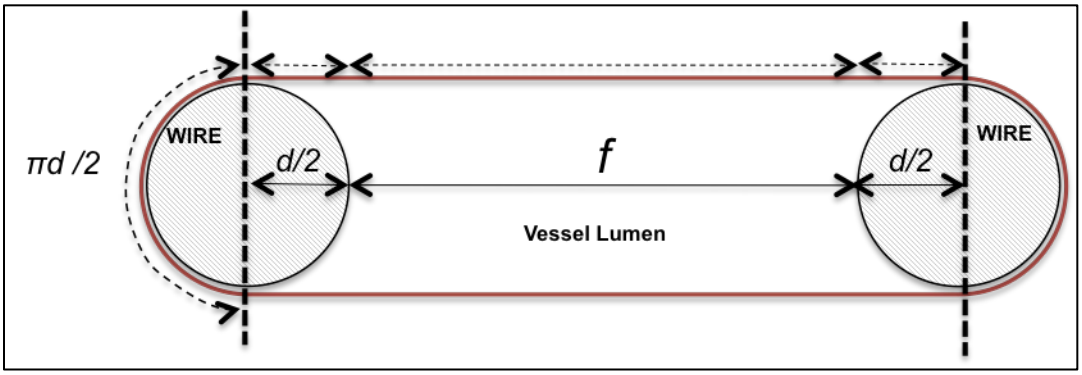

Figure 3 Schematic diagram of a vessel in cross section mounted on two myograph wires. *d* is wire diameter, and *f* is the distance between the wires. These measurements are used to calculate vessel internal circumference (L) from the formula $L = (\pi + 2)d + 2f$

CHPATER 2

Lung region and racing affect mechanical properties of equine pulmonary microvasculature

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J Appl Physiol (1985). 2014 Jun 12. doi: jap.00314.2014. [Epub ahead of print]

Abstract

Exercise-induced pulmonary hemorrhage (EIPH) is a performance-limiting condition of racehorses associated with severe pathology, including small pulmonary vein remodeling. Pathology is limited to caudodorsal (CD) lung. Mechanical properties of equine pulmonary microvasculature have not been studied. We hypothesized that regional differences in pulmonary artery and vein mechanical characteristics do not exist in control animals; and that racing and venous remodeling impact pulmonary vein mechanical properties in CD lung. Pulmonary arteries and veins (range of internal diameters 207 – 386 ± 67 (mean ± s.d.) μm) were harvested from 8 control and 7 raced horses. Using a wire myograph CD and cranioventral (CV) vessels were stretched in 10 μm increments. Peak wall tension was plotted against changes in diameter (length). Length-tension data were compared between vessel type, lung region, and horse status (control and raced). Pulmonary veins are stiffer-walled than arteries. CD pulmonary arteries are stiffer than CV arteries, while CV veins are stiffer than CD veins. Racing is associated with increased stiffness of CD pulmonary veins, and, to a lesser extent, CV arteries. For example, at 305 μm, tension in raced and control CD

veins is 27.74 ±2.91 and 19.67 ± 2.63 mN/mm respectively (mean ± s.e.m; $p < 0.05$, Bonferroni's multiple comparisons test after 2-way ANOVA); and 16.12 ± 2.04 and 15.07 ± 2.47 mN/mm in raced and control CV arteries. This is the first report of an effect of region and/or exercise on mechanical characteristics of small pulmonary vessels. These findings may implicate pulmonary vein remodeling in EIPH pathogenesis.

Introduction

Exercise-induced pulmonary hemorrhage (EIPH) is defined as the presence of frank blood (of pulmonary origin) in the airways after a bout of intense exercise (150, 152, 162). The condition has been reported in several athletic species, including humans (52), racing dogs (42) and camels (4), however it is most commonly described in horses (18, 162). The incidence rate of EIPH in racehorses exceeds 75% (18, 172) when diagnosed by tracheo-bronchoendoscopic examination within 30 – 90 minutes of exercise (69). This highly prevalent condition is associated with impaired racing performance in Thoroughbred horses (70).

EIPH pathology is most severe and most common in caudodorsal lung regions, while the cranioventral lung is spared (150-152, 157, 231, 233). The caudodorsal lung is also the region to which blood flow is preferentially distributed in the horse, both at rest (76), and to an even greater degree, during exercise (15). The distribution and severity of the EIPH lesion matches the distribution of pulmonary blood flow (233) .

Remodeling of small (100 – 200 μm O.D.) intralobular pulmonary veins is a consistent histologic feature of the EIPH lesion (231, 233) and is characterized by accumulation of adventitial collagen, and in more severely affected veins, hypertrophy of

the tunica media (231). Pulmonary arteries, and larger pulmonary veins are not affected in a similar manner. Venous remodeling, along with other EIPH-associated histopathologic changes such as interstitial fibrosis and hemosiderin formation, is limited to the caudodorsal lung (231, 233). In randomly sampled sections of EIPH-affected lung, the entire spectrum of lesions never occurs without co-localized venous remodeling, although venous remodeling occurs on its own (233). This suggests that venous remodeling is an early and key feature of the EIPH lesion, and may be central to the pathogenesis of the disease.

Little is known about regional differences in mechanical characteristics of pulmonary microvasculature in horses, or indeed in any other species. To further understand the pathogenesis of EIPH, a region-specific condition, investigations into regional differences in vascular biology are warranted.

It has been reported that pulmonary vascular remodeling, including collagen deposition, alters the mechanical properties of vessels (101, 207, 212). If pulmonary vein remodeling, such as that observed in EIPH-affected lungs (231, 233) affects wall mechanics, this will have functional ramifications on upstream capillaries. Pulmonary capillary stress failure has been described in the lungs of horses with EIPH (230) and is widely considered to be the source of airway hemorrhage, occurring secondary to dramatic increases in pulmonary vascular pressures in the exercising horse. Pulmonary capillary pressures (which are determined by arterial and venous pressures) are estimated to range between 72 (106) and 83 (127) mmHg during galloping, and transmural pressures in excess of 75 mmHg exceed the breaking strength of equine pulmonary capillaries (17). Remodeled veins are thick-walled compared to normal, unaffected veins, and in some cases have

reduced luminal area (38). Should these changes reduce venous compliance (i.e. increase venous wall stiffness) it follows that, during strenuous exercise, pulmonary capillary pressure will increase yet further and potentially augment EIPH.

The purpose of the study reported here was to test two hypotheses. First, mechanical properties of small pulmonary arteries and veins do not differ by region (cranioventral compared to caudodorsal) in control, unraced horses. Second, pulmonary veins, but not arteries from horses that have a recent racing history have increased wall stiffness compared to veins from horses that have never raced, and this change is limited to veins in the caudodorsal lung, the site of venous remodeling. Wire myography (62) was utilized to evaluate vessel mechanics in these experiments as the equipment is custom-designed for small diameter vessels such as those of interest in this study.

Our data demonstrate regional differences in vessel wall stiffness in both pulmonary arteries and veins from control, unraced horses. Furthermore, caudodorsal veins from raced horses are stiffer than those from control, unraced horses. This finding establishes the first link between descriptions of pulmonary vein remodeling (231, 233) in the horse lung, and the physiologic effects this change is proposed to exert on the pulmonary vasculature during exercise.

Materials and Methods

Animals

For this study, 8 control horses, and 7 raced horses were acquired by donation. Control horses (3 geldings, 5 sexually intact females, 6.6 ± 0.6 (age ± s.e.m.) years) were of various breeds (2 Arabians, and 1 each of Thoroughbred, Standardbred, paint, Quarterhorse,

Hafflinger and crossbred) and did not have a race history. Race-trained horses (3 geldings, 1 sexually-intact male and 3 sexually intact females, 6.3 ± 0.6 (age ± s.e.m.) years) were all Thoroughbreds with a race record. The time period between the last race and euthanasia was 305 ± 77 (mean ± s.d.) days. Raced horses had on average, 22 ± 18 race starts (mean ± s.d., range: 1 – 54 race starts) and were donated for reasons other than severe EIPH (predominantly career-limiting lameness). The Michigan State University Institutional Animal Care and Use Committee approved all experimental procedures.

Tissue acquisition

Horses were administered intravenous heparin sodium (50,000 IU/horse) approximately 15 minutes prior to euthanasia, which was carried out with pentobarbital sodium (90 mg/kg IV). Lung tissue sections (approximately 4 cm^3) were immediately harvested from both the caudodorsal (CD) and cranioventral (CV) regions of the caudal (diaphragmatic) lobe of both left and right lungs. Lung tissue was placed in chilled normal saline (0.9% sodium chloride) solution for transportation to the laboratory. Additional tissue from CD regions of lungs of raced horses was placed in 10% neutral buffered formalin for fixation and histologic assessment.

Vessel dissection

Fresh, chilled lung tissue was sectioned (approximately 0.5 cm thick slices) and pinned to a Sylgard (Dow Corning, Midland, MI) pad in the bottom of a water-jacketed dissection chamber (Radnoti, Monrovia, CA) and fully immersed in chilled Ca^{2+} -free physiologic saline solution (PSS) containing (in mM) 140 NaCl, 5 KCl, 1 $MgCl_2$, 10 HEPES, 10 glucose (pH 7.4,

295 mOsm). A low calcium environment was chosen to minimize vasospasm that can occur during vessel manipulation.

Sections of pulmonary veins and arteries ranging in length from 0.34 to 1.39 mm and between 100 and 400-μm diameter were carefully dissected from tissue based on the following anatomic criteria: intralobular pulmonary veins that course completely alone in the parenchyma(231); pulmonary arteries were collected from broncho-vascular triads. Within a triad of pulmonary artery, vein and conducting airway, pulmonary arteries were easily distinguishable from the vein in the same bundle. They were stiff-walled (compared to veins), and always immediately adjacent to a conducting airway, while veins in the bundle were more distant from the airway (175). Individual vessels were kept in Ca^{2+}-free PSS at 4°C for up to 24 hours until mounted on the myograph.

Wire myography

Vessels were mounted as a cylinder on 2 stainless steel 40-μm diameter wires and the wires secured (one to a micrometer screw and the other to a force transducer) in a 4-chamber myograph (DMT, Aarhus, Denmark). Each chamber contained 5 ml of Ca^{2+}-free PSS, and all vessels were submerged throughout the experiment. The bath fluid was heated slowly to 37°C, and air was bubbled gently through the fluid continuously. Vessel length was recorded using a previously calibrated stereomicroscope, and the micrometer reading at which the wires were barely touching and parallel to one another was recorded. The myograph force transducer was used in conjunction with a PowerLab (ADInstruments, Colorado Springs, CO) data acquisition unit and LabChart (ADInstruments, Colorado Springs, CO) software platform.

Wires were then separated by use of the micrometer until the transducer registered a small (<0.05 mN) but sustained force. The micrometer reading was again recorded, and this was designated an individual vessel's start point. Vessel diameter was calculated and recorded at this point. From that point, wires were separated from one another in 10-μm increments. The peak force achieved at each micrometer adjustment was recorded, and once a plateau in force was attained the wires were separated again for the next force measurement. Vessels were stretched in this stepwise manner until stretching resulted in no further increase, or a decrease in force was recorded, which was interpreted as vessel failure.

Vessel histology

Once length-tension data acquisition was complete, a subset of vessels was fixed *in situ* on the myograph wires in 10% neutral buffered formalin. Following fixation, the vessels were removed from the wires and mounted orthogonal to the long axis in Histogel (American MasterTech, Lodi, CA) specimen processing gel. The gel-embedded vessel was then embedded in paraffin for sectioning. 6-μm sections were placed on glass slides and stained with hematoxylin and eosin (H and E) and Verhoeff-Van Gieson (VVG). These stained sections were used to confirm vessel identity as a pulmonary artery, or a pulmonary vein. Slides were reviewed by a board-certified veterinary pathologist (KJW) who was blinded to the identity of the vessel based on anatomic/dissection criteria. Pulmonary arteries were identified based on a relatively substantial *tunica media* that was bounded by both an internal and external elastic lamina (134). In contrast, pulmonary veins had less smooth muscle in the *tunica media,* a single distinct external elastic lamina between the *tunica*

media, and *tunica adventitia* (134) and an absent internal elastic lamina between the *tunica intima* and *tunica media* (175, 210)**(Figure 4).**

Lung histopathology

Fixed lung tissue samples from the CD regions of 6 of the 7 raced horses underwent routine processing and embedding in paraffin for histological examination. Following sectioning, tissues were stained with H and E and VVG, and evaluated by KJW for the presence and severity of EIPH vascular pathology using previously described criteria (231).

Data Analysis

Variability between vessels was similar to variability between animals, therefore, in this study, the sampling units are individual vessels, and not horses.

Vessel internal diameters at the start point were calculated as follows: vessel internal circumference at that point (C) divided by π. Vessel internal circumference (C) was calculated as twice the distance between the wires, plus the wire circumference plus twice the wire diameter (242). The effect of vessel type (artery or vein) and region (caudodorsal or cranioventral) on diameter at the start point was analyzed within racing status (control or raced) using a two-way ANOVA with Bonferroni's multiple comparisons test (GraphPad Prism 6, GraphPad Software Inc., La Jolla, CA).

Change in vessel internal diameter from that vessel's diameter at start point (as previously defined) was referred to as length (L) and expressed in μm. L for each vessel was plotted against recorded tension (T). T was defined as the peak force registered per unit of vessel segment length, and expressed in mN/mm. Vessels were analyzed over a

range of L that did not cause failure in any vessel (0 - 337.41 μm). Length-tension data were compared (a) between regions (caudodorsal and cranioventral), (b) between vessel type (artery and vein), and (c) between racing status (control and raced) within region and vessel type using a 2-way ANOVA with Bonferroni's multiple comparisons test (GraphPad Prism 6, GraphPad Software Inc., La Jolla, CA).

In order to evaluate changes in mechanical properties within a physiologically relevant range, tension (T) values were converted to equivalent transmural pressure values (P) by application of the Laplace relationship as follows:

$$P_i = T_i / (C_i/2 * \pi)$$

where P_i, T_i and C_i are pressure, tension and internal circumference respectively at a given length value, *i*. Over the physiologic range of pressures encountered in arteries and veins, pressure (P) values in mN/mm^2 were converted to mmHg, and plotted against the change in vessel diameter from start point, or length (L). Length-pressure data from arteries and veins were compared between racing status (control and raced) within each region (CD and DV) using a 2-way ANOVA with Bonferroni's multiple comparisons test (GraphPad Prism 6, GraphPad Software Inc., La Jolla, CA).

Results

From control horses, 9 veins and 10 arteries were harvested from caudodorsal lung regions, and 7 veins and 9 arteries from cranioventral lung. From raced horses, it was 15 veins and 10 arteries from caudodorsal lung regions, and 8 veins and 10 arteries from cranioventral lung. Seventy-eight vessels were included in all subsequent statistical analyses.

Vessel diameters

Vessel diameters are shown in **Table 1**. Diameters did not differ between arteries and veins from caudodorsal and cranioventral lung in both control and raced horses with the following exception: arteries from the caudodorsal lung region were larger in diameter than veins from both caudodorsal and cranioventral lung region of raced horses.

Vessel identification

Histology was performed on 29 of the 78 vessels that were studied. All vessels that were identified during dissection as either an artery or a vein had that identity confirmed by use of histology **(Figure 4).**

Length-tension data

When data from the CD and CV vessels were combined, pulmonary veins (n = 16 and n = 23 for control and raced respectively) were stiffer (as demonstrated by a steeper length-tension curve) than pulmonary arteries (n = 19 and n = 20 for control and raced respectively). This observation was consistent in both control and raced horses (**Figure 5**, A and B respectively) ($p < 0.0001$).

In control horses, pulmonary veins from the cranioventral lung were stiffer than those from the caudodorsal lung ($p < 0.0001$), whereas pulmonary arteries from caudodorsal lung were stiffer than arteries from cranioventral lung ($p < 0.0001$) (**Figure 6**, A and B respectively). This regional pattern of differences in vessel stiffness was maintained in vessels from raced horses (**Figure 6**, C and D).

Vessels from raced horses were compared to vessels from control horses within lung region. Cranioventral veins and caudodorsal arteries were not affected by race training ($p = 0.8078$ and $p = 0.4317$ respectively) (**Figure 7**, B and C respectively). However caudodorsal veins from raced animals were significantly stiffer than those from control animals ($p < 0.0001$) (**Figure 7**, A). Cranioventral arteries from raced horses were also significantly stiffer than those from control horses ($p = 0.0014$) (**Figure 7**, D).

Length-pressure data

Tension values were converted to equivalent transmural pressure values encompassing *in vivo* physiologic pressure ranges experienced by horses at rest and during exercise: 0-120 mmHg for arteries, and 0 – 80 mmHg for veins. Length-pressure data in these ranges were compared between control and raced horses within lung region. Cranioventral veins and caudodorsal arteries were not affected by race training ($p = 0.9416$ and $p = 0.0552$ respectively) (**Figure 8**, B and C respectively). However caudodorsal veins and cranioventral arteries from raced animals underwent a significantly smaller change in internal diameter over a physiologic pressure range than vessels from control animals ($p < 0.0001$) (**Figure 8**, A and D respectively).

Lung histopathology

Venous remodeling and lung pathology consistent with exercise-induced pulmonary hemorrhage changes were detected in all caudodorsal lung sections examined from raced horses. The venous remodeling was consistent with that previously described in association with EIPH (231, 233). Briefly, these changes consisted of mild to moderate

increases in adventitial collagen surrounding small veins along with small numbers of hemosiderophages, indicating prior hemorrhage and erythrophagocytosis by the alveolar macrophages.

Discussion

To the authors' knowledge, this is the first published account of the effect of lung region, and/or exercise on pulmonary microvascular mechanical properties in any species to date. Study of these factors in equids is particularly important in order to better understand exercise-induced pulmonary hemorrhage (EIPH) pathogenesis.

The predominant tissue types that determine a vessel's mechanical characteristics are collagen and elastin (20, 177). Elastin contributes most resistance to stretching at lower tension values, whereas collagen provides most resistance at higher tension values (20, 177). Collagen is minimally distensible, and has an elastic modulus that is approximately 400 times that of elastin (20).

In both control and raced horses, pulmonary veins are stiffer-walled than pulmonary arteries, and we propose that this difference is due to the greater proportion of collagen in vein walls, compared to arteries. This particular finding has been documented in other species. In the dog, intraparenchymal pulmonary veins are less distensible in response to increases in intravascular pressure than equivalently-sized pulmonary arteries (121). Larger pulmonary veins are also less distensible than pulmonary arteries in both rabbits (22), and in humans (9, 119).

Mechanical properties of small-caliber pulmonary vessels such as those evaluated in this study are not well described in the literature to date, and to the best of the authors'

knowledge, published data regarding regional differences in mechanical properties of pulmonary arteries and veins of this size range in any species do not exist. In both control and raced horses, caudodorsal arteries are stiffer-walled than cranioventral arteries. Due to the absence of similar observations in other species, this finding is unexpected, and any proposed rationale for this observation at this time remains speculative. However, in light of what is known about pulmonary blood flow distribution in quadruped species, it is considered likely that these differences are related to the inhomogeneous blood flow distribution patterns that are observed in the horse (76) and in other mammals (56, 57). Stiffer-walled pulmonary arteries in caudodorsal lung may represent an adaptive response to normal preferential distribution patterns of blood flow to this region, which in turn is largely due to the anatomy of the pulmonary vascular system (77). This change may also serve to protect pulmonary capillaries in this region from a higher-flow state during intense exercise.

In both control and raced horses, cranioventral veins are stiffer-walled than caudodorsal veins. Considering an example from the equine systemic circulation, veins within the foot (i.e. laminar veins) are exposed to the highest intravascular pressures in the limb due to gravitational forces, and are thick-walled (6) structures that are difficult to discern from laminar arteries (97). It is possible that pulmonary veins from the cranioventral lung are stiffer-walled than their caudodorsal counterparts due to a similar adaptive response to the hydrostatic pressure difference between the dorsal and ventral lung.

Although vessel mechanics play a role in influencing blood flow distribution, vessel reactivity must also be considered a key determinant of pulmonary blood flow.

Interestingly, a small number of studies report regional differences in pulmonary vessel reactivity, both in the horse (164) and in the pig (51, 176). Larger dorsal arteries in both pigs (176), and horses (164) demonstrate enhanced endothelial-mediated vasorelaxation compared to vessels from ventral regions. Regional patterns of vessel mechanical properties, along with vascular reactivity merit further and more detailed investigation.

An important finding of this study was that caudodorsal veins from raced animals were significantly stiffer than those from control animals. This increase in stiffness is most likely a consequence of venous remodeling in raced horses. We previously reported that small pulmonary veins in caudodorsal, but not in cranioventral lung remodel in EIPH-affected horses, while equivalently-sized pulmonary arteries are largely unaffected in both regions (231). EIPH-associated venous remodeling is typified by extensive adventitial collagen expansion (231) resulting in reduced lumen area (38). In studies performed on the pulmonary vasculature of other species, remodeling is also associated with altered mechanical properties (101, 207, 212).

An increase in stiffness of pulmonary veins has important physiologic consequences. Increased vein stiffness will decrease pressure-induced distension of the veins and increase resistance to blood flow, particularly during exercise. This, in turn, should increase pressure in upstream pulmonary capillaries. If pressure increases exceed the reported breaking strength of these vessels (P_{tm} 75 mmHg (17)), stress-failure (230) of capillary walls can occur, resulting in EIPH.

Despite the difference between length-tension curves of pulmonary arteries harvested from cranioventral lung in raced and unraced horses being small, this difference was statistically significant. The observation that cranioventral pulmonary arteries were

stiffer in raced horses was unexpected, as vascular pathology in cranioventral lung is not reported in existing literature on the topic (152, 157, 231). As extensive EIPH pathology in this region is not observed, it is possible that mild arterial remodeling changes have been overlooked in past studies and further more detailed studies of the vasculature of this region are warranted based on this observation. It is plausible that stiffening of cranioventral pulmonary arteries in response to race training is somewhat protective of capillaries in that region, and that through this mechanism, this observation may also explain (at least in part) the regional nature of the EIPH lesion.

Alterations in vessel stiffness, as determined by analysis of length-tension curves, are a direct result of changes in vessel wall structural components (119). In this study, we evaluated tension over a wide range, from zero-stress to tension values approaching vessel breaking point. In light of previous studies in EIPH-affected horses that report collagen deposition in remodeled vein walls (38, 231, 233) it is most likely that the observed increase in vessel stiffness is due to an increase in wall collagen content. In this study all vessels were stretched to near-breaking point, which distorted vessel wall anatomy greatly. For this reason, morphometric analyses of the vessels were not performed, and therefore we do not provide data quantifying collagen deposition in affected vessels. Studies using a reduced range of tension application to correlate collagen content and mechanical properties are warranted.

Vessel mechanics were analyzed over a large range of wall tensions, which undoubtedly exceeds any wall tension changes experienced during exercise *in vivo.* Extrapolation of the effect of mechanical changes over such a large wall tension range on vessels in the living horse at rest or during exercise is difficult, and predicted effects, if any,

should not be overstated. In an effort to address this, we converted tension values to equivalent transmural pressure values using the Law of Laplace, and evaluated length-pressure relationships over a range encompassing *in vivo* physiologic pressure ranges experienced by horses at rest and during exercise: 0-120 mmHg for arteries, and 0 – 80 mmHg for veins (127). This analysis demonstrated an identical pattern of significant effects of racing (stiffening of caudodorsal veins and cranioventral arteries) to that found in the larger (supra-physiologic) range of vessel wall tensions. It is reasonable to extrapolate the effect of these data to vessels *in vivo,* and these findings lend further support to the hypothesis that pulmonary venous remodeling in the caudodorsal lung, which in this study manifests as an increase in vessel wall stiffness, may be a component of EIPH pathogenesis.

Previous reports of EIPH pathology have utilized horses with career-limiting EIPH (157, 231, 233) and correspondingly severe pulmonary pathologic changes. In contrast, raced horses used in this study were retired from racing for reasons other than EIPH. Interestingly, and despite an absence of severe EIPH clinical history, these horses had mild to moderate EIPH pathology, suggestive of underlying prior pulmonary hemorrhage. Consistent with previous reports (231, 233), caudodorsal veins of raced horses in this study were remodeled. The data presented in this paper expand this observation by demonstrating that this remodeling is associated with increased vein wall stiffness. These alterations in vessel mechanics further substantiate the contention that remodeling of the pulmonary venous system of the equine lung is an early response to strenuous exercise, The authors propose that this remodeling may have a role in the pathogenesis of EIPH (233) although it is acknowledged that the experiments described in this study were not designed to ascertain whether alterations in pulmonary vein stiffness contribute to EIPH,

or, whether the changes come about because of EIPH earlier in these horses' racing careers. Further support for a relationship between histologic remodeling changes and increased vein stiffness is provided by the observation that the mechanical properties of cranioventral veins and caudodorsal arteries, vessels that are not reported to remodel in EIPH-affected horses (231, 233), were unaffected by racing.

In conclusion, the findings of this study indicate for the first time, that regional differences in vessel mechanics exist in the unraced horse, and that changes in pulmonary vein wall structure occur in horses that have undergone race training. Furthermore, these changes occur before the development of severe, career-limiting EIPH. Altered vessel mechanics are detectable within a physiologically applicable range of vessel wall tensions. Therefore this finding may have important consequences in the exercising horse. Increased vein wall stiffness increases pulmonary capillary pressures, particularly during exercise, thereby augmenting EIPH. These data highlight pulmonary vein remodeling as a lesion of interest in EIPH pathogenesis, and suggest that pulmonary veins could be an interesting therapeutic target in future approaches to EIPH management.

APPENDIX

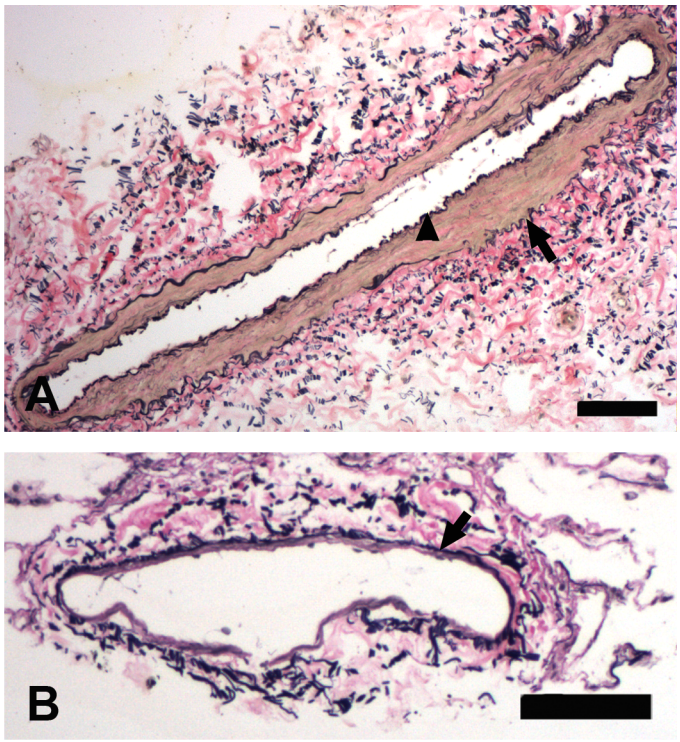

Figure 4 Verhoeff-Van Geison stained pulmonary artery and vein for histologic confirmation of identity post-myography. Pulmonary arteries (A) are thicker walled than veins (B) and possess both an internal (arrowhead) and external (arrow) elastic lamina compared to veins which are thin-walled and only have an external elastic lamina (arrow). Bar = 100 μm.

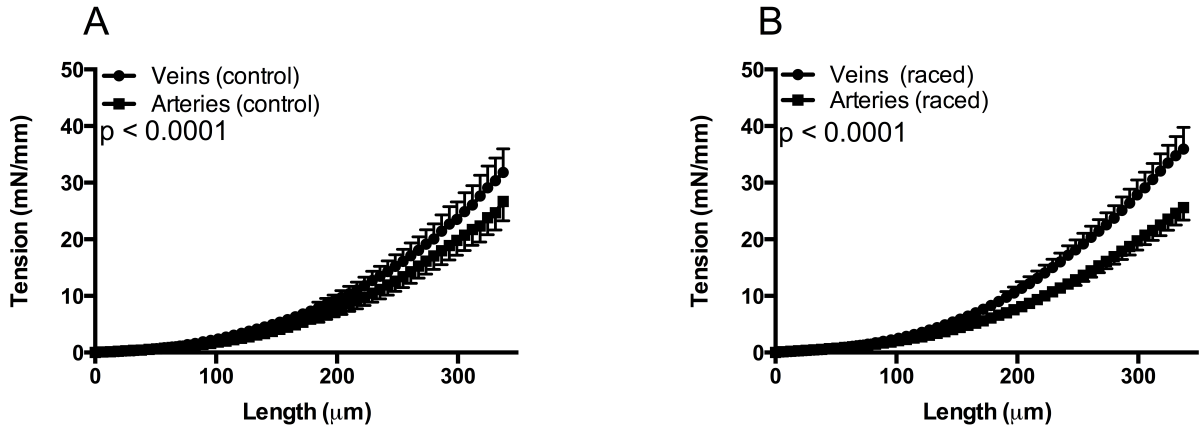

Figure 5 Length-tension plots for arteries and veins from control, unraced (A) and raced (B) horses. Values are mean ± SE. In both control and raced horses, veins are stiffer than arteries. $P < 0.05$ is considered significant.

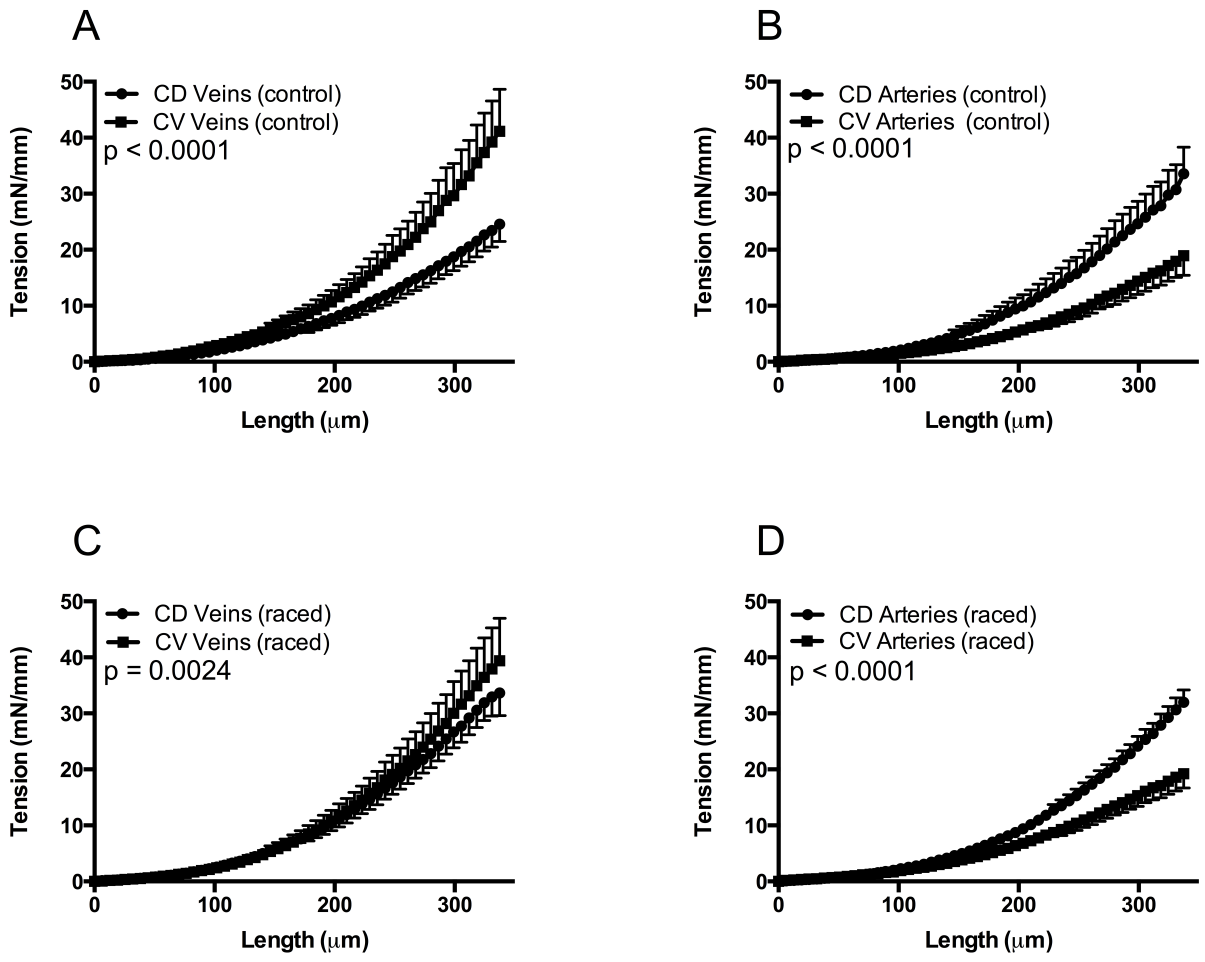

Figure 6 Length-tension plots for caudodorsal (CD) and cranioventral (CV) veins, and for CD and CV arteries from unraced horses (A and B respectively) and from raced horses (C and D respectively). Values are mean ± SE. In both control and raced horses, veins from cranioventral lung are stiffer than veins from caudodorsal lung, and arteries from caudodorsal lung are stiffer than arteries from cranioventral lung. $P < 0.05$ is considered significant.

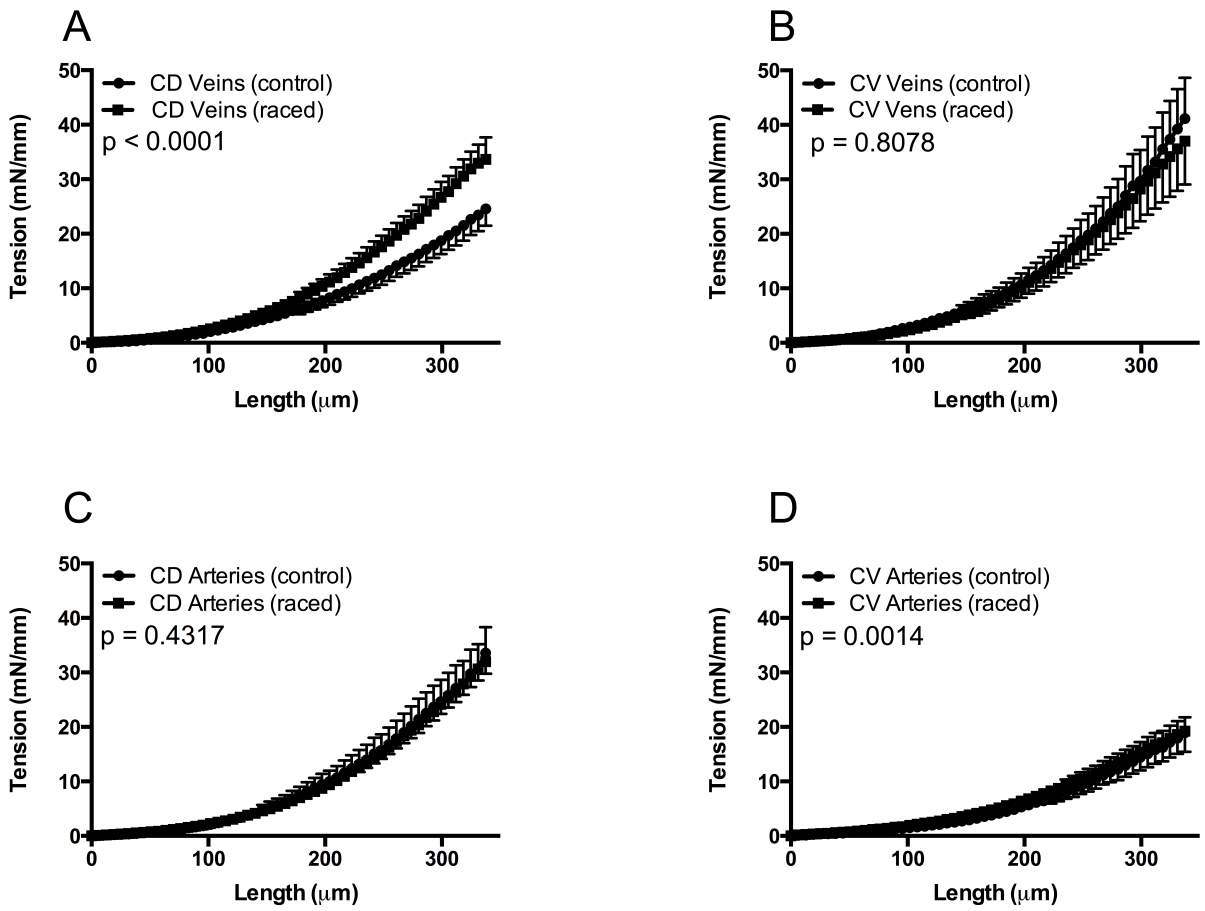

Figure 7 Length-tension plots of vessels from control and raced horses. Data are from caudodorsal (CD) and cranioventral (CV) veins (A and B respectively) and arteries (C and D respectively). Values are means ± SE Veins from the caudodorsal region of lungs of raced horses are stiffer than those from unraced, control horses. Arteries from the cranioventral region of lungs of raced horses are stiffer than those from unraced, control horses. $p < 0.05$ is considered significant.

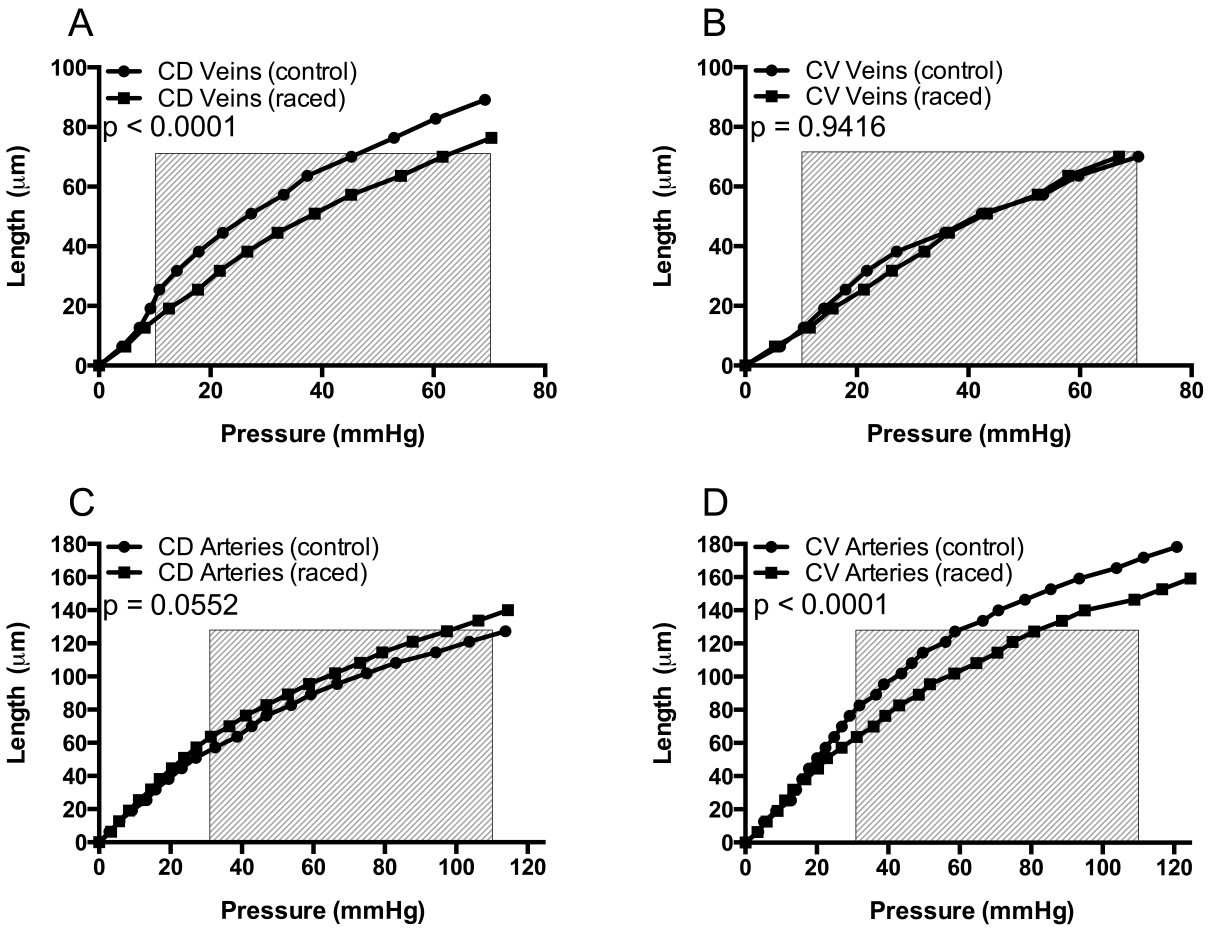

Figure 8 Length-pressure plots for vessels from control and raced horses. Data are from caudodorsal (CD) and cranioventral (CV) veins (A and B respectively) and arteries(C and D respectively). Data are expressed as mean values. Veins from the caudodorsal region of lungs of raced horses are stiffer than those from unraced, control horses. Arteries from the cranioventral region of lungs of raced horses are stiffer than those from unraced, control horses. Hatched regions demarcate the range of *in vivo* intravascular pressures from rest to intense exercise (10 – 70 mmHg and 30 – 110 mmHg for veins and arteries respectively). $P < 0.05$ is considered significant.

Vessel Type	Region	Horse Status	n	Diameter at start (µm) mean ± s.e.m
Vein	CD	Control	9	217.5 ± 25.21
Artery	CD	Control	10	280.0 ±38.81
Vein	CV	Control	7	207.3 ± 30.83
Artery	CV	Control	9	314.5 ± 37.42
Vein	CD	Raced	15	223.8 ± 15.35
Artery	CD	Raced	10	386.3 ± 29.28
Vein	CV	Raced	8	211.9 ± 34.68
Artery	CV	Raced	10	330.3 ± 27.2

Table 1 Mean vessel diameters at start point. Values are means ± SE, n = number of vessels. Start point is defined as the smallest diameter at which a vessel maintains a small (<0.05 mN) but sustained wall tension. CD, caudodorsal; CV cranioventral

CHAPTER 3

Regional heterogeneity in reactivity of small pulmonary blood vessels in the horse may predict exercise-induced pulmonary hemorrhage lesion distribution

Alice Stack, Frederik J. Derksen, Kurt J. Williams, N. Edward Robinson, William F. Jackson

Abstract

Exercise-induced pulmonary hemorrhage in horses results in significant caudodorsal (CD) lung region pathology. Capillary stress failure and hemorrhage occur secondary to high pulmonary circulation pressures during exercise, but reasons for CD EIPH lesion distribution have not been established. Alterations in vascular tone of small pulmonary arteries and veins impact pulmonary capillary pressures. We investigated the hypothesis that regional heterogeneity in active control mechanisms of small pulmonary vessels exist, and do so in a manner that predicts EIPH lesion distribution. Autonomic control of vascular tone changes during exercise, therefore the vasoactive autonomic agonists phenylephrine, isoproterenol and methacholine were investigated using wire myography on vessels dissected from CD and cranioventral (CV) regions of 12, unraced horses. U 46619, furosemide, and mechanisms of methacholine activity using L-NAME and indomethacin pre-incubation steps, were also investigated. Phenylephrine did not cause contraction in any vessels, whereas isoproterenol relaxed pre-contracted arteries (CD to a greater degree than CV) but not veins. Methacholine caused contraction of CD arteries, and relaxation of CV arteries and all veins in a non-region dependent manner. L-NAME and indomethacin inhibited methacholine-induced relaxation of CV arteries, whereas indomethacin only

augmented CD artery contraction. Furosemide caused mild relaxation of pulmonary arteries and veins at high concentrations. Extrapolation of these data to the *in vivo* effect of increased sympathetic and decreased parasympathetic tone during exercise predicts that highest capillary pressures occur in CD lung, explaining in part EIPH lesion distribution and disease pathogenesis. Regional heterogeneity in small pulmonary vessel reactivity is unreported in other species.

Introduction

Exercise-induced pulmonary hemorrhage (EIPH), which is diagnosed when blood is identified in the airways after an intense bout of exercise(162), has been reported in multiple athletic species (1, 10), including humans(4, 42, 52), but it is most prevalent in racehorses (18) where it is associated with impaired racing performance(70).

An early theory of EIPH pathogenesis is based on descriptions of capillary wall disruption in lung tissue from exercised horses(230), that is likely a result of the high intravascular pressures that occur normally in the pulmonary vascular system of the exercising horse (i.e. capillary stress failure). High pulmonary artery and venous pressures result in capillary pressures in the order of 80 mmHg (106, 127), exceeding the reported threshold for breaking strength (75 mmHg) of equine pulmonary capillaries (17).

Stress-failure alone does not account for the pathology in lungs of EIPH-affected horses however (150, 233). EIPH lesions include remodeling of small (100 – 200 μm O.D.) pulmonary veins (231), and are most common and most severe in the caudodorsal lung region while cranioventral lung remains normal(150, 157, 231, 233). Pulmonary venous

remodeling is reported as a consequence of elevated intravascular pressures in other species (25, 86), and is predicted to occur in response to elevated venous pressures in the exercising horse, particularly in the caudodorsal lung.

Caudodorsal lung is the region to which blood is preferentially distributed in both the standing (76), and galloping horse (15) and we recently confirmed that EIPH pathology has a similar distribution to that of pulmonary blood flow (233). Blood flow distribution in the lung is determined by vascular anatomy, which is fixed, and variable factors including vascular reactivity (55). Regional differences in endothelial-dependent reactivity of large pulmonary arteries of the horse that can account for this flow distribution pattern have already been reported (164), however reactivity patterns of small pulmonary vessels have not. These vessels are worthy of investigation as arterial and venous resistances in the small vessels supplying and draining the capillaries directly affect pulmonary capillary pressure, the ultimate cause of stress-failure and hemorrhage. For example, reduced arterial resistance and/or increased venous resistance will further increase pulmonary capillary pressure, thereby augmenting the risk of EIPH.

In general, sympathetic nervous system activation associated with exercise results in both α- and β- adrenoreceptor activation in the pulmonary circulation (93, 203), while parasympathetic activity during exercise is diminished (133). Accounts of regional heterogeneity in reactivity profiles of large pulmonary vessels, and mechanical properties of small pulmonary vessels in the horse exist (164, 196). Regional differences in vascular responses to increases and/or decreases in vasoactive autonomic agonists would exert varying effects on pulmonary capillary pressures in the lung.

For these reasons, we investigated the hypothesis that regional differences in patterns of vascular reactivity to adrenergic and cholinergic agonists in small pulmonary arteries and veins exist, and do so in a manner that will predict the predilection of EIPH pathology for the caudodorsal lung region.

EIPH severity and incidence is ameliorated in part by pre-race administration of furosemide (73), which is a pulmonary venodilator in dogs(59). We also tested the hypothesis that, in horses, furosemide dilates pulmonary veins but not pulmonary arteries independent of lung region.

To test these hypotheses, pharmacology studies using wire myography were performed on small pulmonary arteries and veins from unraced horses. Both adrenergic (phenylephrine and isoproterenol) and muscarinic (methacholine) agonists were tested, along with the thromboxane analog U 46619, and furosemide. Regional differences in responses of pulmonary arteries to isoproterenol and methacholine, and responses of veins to U 46619 are reported. Further investigations into mechanisms of methacholine activity in pulmonary arteries were performed based on observed differences. Regional differences in reactivity profiles of small pulmonary vessels such as those described in these studies have not been reported in any species to date.

Materials and Methods

Animals

Nine horses, (7 geldings and 2 mares), 8.33 ± 0.9 (average age ± s.e.m.) years, were used to determine regional patterns of reactivity to 5 drugs. These horses were of various breeds including two Quarterhorses, two Tennessee Walkers, three Arabian-crosses, one

Thoroughbred and one crossbred. Three additional horses (2 mares and one gelding), 9.67 ± 2.03 (average age ± s.e.m.) years, were used to investigate mechanisms of methacholine activity. These horses were of various breeds including two paint-crosses and one Thoroughbred. Thoroughbred horses had never raced. The Michigan State University Institutional Animal Care and Use Committee approved all experimental procedures.

Tissue acquisition

Approximately 15 minutes before euthanasia, intravenous heparin sodium (50,000 IU/horse) was administered to all horses. Euthanasia was performed using pentobarbital sodium (90 mg/kg IV). Lung tissue sections (approximately 4 cm^3) were acquired from the caudodorsal (CD) and cranioventral (CV) regions of the caudal (diaphragmatic) lobe of both left and right lungs. Lung tissue was kept in chilled normal saline (0.9% sodium chloride) solution until vessel dissection.

Vessel dissection

Approximately 0.5 cm thick slices of lung tissue were pinned to a Sylgard (Dow Corning, Midland, MI) pad in a water-jacketed dissection chamber (Radnoti, Monrovia, CA) and fully submerged in chilled Ca^{2+} -free physiologic saline solution (PSS) containing (in mM) 140 NaCl, 5 KCl, 1 $MgCl_2$, 10 HEPES, 10 glucose (pH 7.4, 295 mOsm). Ca^{2+} -free PSS was used to minimize vasospasm that can occur during vessel manipulation.

Sections of pulmonary veins and arteries (identified by use of anatomic criteria) that ranged in length from 0.38 mm to 1.46 mm were gently dissected from pulmonary tissue. Pulmonary arteries were harvested from broncho-vascular triads. Pulmonary arteries were

identified as stiff-walled vessels (compared to veins in the same triad) that were immediately adjacent/adherent to the conducting airway (175). For this experiment, veins were not harvested from the bronchovascular triad and intralobular pulmonary veins only were harvested. They are found alone in parenchyma, and are not associated with airways (231). Once dissected, vessels were kept in Ca^{2+}-free PSS at 4°C for up to 24 hours.

Wire myography

Vessels were submerged in chilled Ca^{2+}-free PSS and mounted as a cylinder on 2 intra-luminal stainless steel 40-μm diameter wires in a 4-chamber myograph (DMT, Aarhus, Denmark) as previously reported (196). Bath fluid was then exchanged for room temperature PSS containing (in mM) 140 NaCl, 5 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose (pH 7.4, 295 mOsm). Vessel length was recorded using a previously calibrated stereomicroscope. The bath fluid was then heated slowly to 37°C, and air was bubbled gently through the fluid continuously. The myograph force transducer was used in conjunction with a PowerLab (ADInstruments, Colorado Springs, CO) data acquisition unit and LabChart (ADInstruments, Colorado Springs, CO) software platform.

Vessel normalization

Optimal passive tension values for equine pulmonary arteries and veins have not been published. Therefore the first 11 arteries harvested were tested in order to determine this characteristic. Briefly, resting wall tension (T) was set at 0.1 mN/mm, and PSS was exchanged for 60 mM K^+ PSS (which contained (in mM) 85 NaCl, 60 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose (pH 7.4, 295 mOsm)). Vessels were allowed to develop a contraction

for 2 minutes, the maximum T value reached was recorded, and the vessel was washed with PSS until T returned to baseline. After 5 minutes, resting wall T was increased by 0.2 mN/mm (to 0.3 mN/mm) and PSS was exchanged for 60 mM K^+ PSS as before. This process was repeated until an increase in passive wall T no longer resulted in an increase in active T upon the addition of 60 mM K^+ PSS. Pulmonary veins underwent a similar procedure other than passive wall T for veins was increased in 0.1 mN/mm increments.

Once it became apparent that pulmonary arteries had similar, repeatable optimal T values (determined in 11 arteries from 4 horses), mean optimal T was calculated, and this value, 1.1 mN/mm, was used to normalize all subsequent arteries. Veins had a greater range of optimal T values (from 0.2 – 0.7 mN/mm), and therefore this value was determined with 60 mM K^+ PSS for each vein studied in subsequent experiments. Vessel diameter was measured under minimal tension (0.1 mN/mm for arteries and veins) and at optimal tension (1.1 mN/mm for arteries, individual wall tension for veins) conditions.

Vessel wake-up

Once normalized, vessels were allowed a 40-minute equilibration period at their optimal T. Then vessels underwent a wake-up procedure consisting of 2 challenges with 60 mM K^+ PSS with a 5-minute interval.

Agonist concentration-response curves

Cumulative concentration-response curves (CCRC) were generated for each drug, with the exception of phenylephrine for which a single concentration challenge (10^-5 M) was performed. Concentration ranges were initially established based on literature from other

species, and then confirmed in equine vessels during preliminary experiments. U 46619, methacholine and isoproterenol concentrations ranged from 1 x 10^-9 to 3 x 10^-6 M. Furosemide was tested over 10^-5 to 3 x 10^-4 M. DMSO (without furosemide) was tested to rule out vehicle effects.

In order to evaluate responses to vasodilator agents (methacholine, isoproterenol, furosemide), all vessels were first exposed to 10^-6 M U 46619, a concentration that was confirmed as reliably producing maximal vasoconstriction in both arteries and veins during preliminary experiments.

Multiple drugs (up to 4) were tested in the same vessel with thorough washes between each challenge. The order in which drugs were tested on a set of 4 vessels was randomized using a random list generator.

For the second component of this study in which mechanisms of methacholine-reactivity were investigated, artery wall T was set at 1.1 mN/mm. 30 minutes after vessel wake-up, a methacholine CCRC was performed on all vessels. After washing, vessels were incubated with either L-NAME (10^-4 M) or indomethacin (10^-5 M) for 30 minutes, and the methacholine CCRC was repeated. After washing, all vessels were incubated with L-NAME and indomethacin for 30 minutes and a third methacholine CCRC was performed in the presence of both inhibitors.

Vessel fixation

After pharmacology experiments were concluded, vessels were cut along their long axis between the myograph wires. Vessels were then pinned out with the endothelial surface

exposed onto a Sylgard (Dow Corning, Midland, MI) pad in a small petri dish filled with PBS. Vessels were fixed *in situ* using 10% methanol-free formaldehyde for 20 minutes.

Immunohistochemistry

After washing with PBS, immunoflourescent staining for endothelium (CD-31) was carried out on the wholemount vessels from the second component of the study. Monoclonal mouse anti-human CD-31 primary antibody (Dako, Carpinteria, CA) (1:40) was applied to the vessels and incubated overnight at 4°C. After blocking (with 5% normal goat serum in a 1% saponin in PBS solution) for 1 hour, Alexa Fluor 488-conjugated AffiniPure goat anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) (1:100) was applied for 1 hour. Vessels were then mounted on slides under PBS.

Endothelial Imaging

Endothelium was examined using epiflourescent microscopy. Each vessel was photographed at 20X magnification, and depending on vessel surface area, between 2 and 7 images per vessel acquired and saved. Endothelium-covered regions were outlined using imaging software (Image J, http://imagej.nih.gov/ij/) and total endothelium-covered area expressed as a percentage of the total tissue area in an image.

Vessel histology

Following fixation or imaging, vessels were removed from the petri dish or slide and placed in Histogel (American MasterTech, Lodi, CA) specimen processing gel. Gel-embedded vessels were embedded in paraffin and sectioned. 6-μm sections were stained with

hematoxylin and eosin (H and E) and Verhoeff-Van Gieson (VVG), and stained tissues were used to confirm vessel identity as either a pulmonary artery or vein. Characteristics of pulmonary arteries include a substantial *tunica media* bounded by an internal and external elastic lamina (134). In contrast, pulmonary veins have less smooth muscle and only an external elastic lamina between the *tunica media* and *tunica adventitia* (134). The internal elastic lamina between the *tunica intima* and *tunica media* (175, 210) is absent. Vessel identification was carried out by a board-certified veterinary pathologist (KJW) who was blinded to the identity of the vessel based on anatomic/dissection criteria.

Materials

U 46619 was acquired from Tocris Bioscience (Minneapolis, MN). Acetyl-β-methylcholine chloride, phenylephrine hydrochloride, isoproterenol hydrochloride, furosemide, N_{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME) and indomethacin were acquired from Sigma-Aldrich (St. Louis, MO). U 46619 and furosemide were dissolved in DMSO, indomethacin was dissolved in 20X bicarbonate buffer, and all other drugs were dissolved in double-distilled water to make stock solutions. Serial dilutions of stock solutions were made in PSS.

Statistical analyses

Responses of a vessel to vasoconstrictor agents (U 46619 and phenylephrine) were expressed as a percentage of the mean of that vessel's maximal contractions in response to 60 mM K^+ -PSS during vessel wake-up.

Once a stable contraction to 10^-6 M U 46619 was established, all responses to vasodilator agents (methacholine, isoproterenol and furosemide) were expressed as a percentage of that contraction.

Cumulative concentration response curves were fit (when possible) to a log(agonist) vs. response sigmoidal curve (Y=Bottom + (Top-Bottom)/(1+10^((LogEC50-X)*HillSlope))) and curve fits were then compared between arteries and veins, and between lung regions (GraphPad Prism 6, GraphPad Software Inc., La Jolla, CA). EC50 is that concentration of agonist that gives a response halfway between the bottom and the top (plateau regions) of the sigmoidal curve. All data are expressed as mean ± s.e.m. and $p < 0.05$ is considered significant.

For image analysis, percent endothelium-cover in an image was averaged for each vessel. Mean percent endothelium-cover for CD and CV vessels was compared using an unpaired t-test (GraphPad Prism 6, GraphPad Software Inc., La Jolla, CA) and statistical significance declared at $p < 0.05$.

Results

Vessels

Twenty-nine pulmonary arteries (13 from CD and 16 from CV lung regions) and 23 pulmonary veins (11 from CD and 12 from CV lung regions) from 9 horses were used to study regional patterns of reactivity to drugs, and 30 pulmonary arteries (15 from CD and 15 from CV lung regions) from 3 different horses were used to investigate mechanisms of methacholine activity.

Vessel dimensions

Diameter of arteries and veins under minimal tension of 0.1 mN/mm was 169.9 ± 10.91 (mean ± s.e.m.) and 128.3 ± 9.0 μm, respectively. Under optimal tension arterial diameter was 367 ± 16.23 (mean ± s.e.m.) μm, while venous diameter was 205.8 ± 20.86 (mean ± s.e.m.) μm. Using the Law of Laplace to convert wall tension (in mN/mm) to transmural pressure values (in mmHg), optimal wall tension values (1.1 mN/mm for arteries, and 0.32 mN/mm for veins) were equivalent to pressures of 24.21 ± 1.1 and 14.05 ± 1.4 (mean ± s.e.m.) mmHg in arteries and veins respectively.

U 46619

U 46619 caused concentration-dependent contraction in both arteries (n = 16) and veins (n = 15) (**Figure 9**, A), with the greatest response observed in veins. The maximum response of veins was 234.2 ± 15.06 % of maximum response to KCl, whereas that of arteries was 104.5 ± 4.89 %. Pulmonary veins were more sensitive to U 46619 than pulmonary arteries with EC50 values of 9.74 x 10^-8 M and 2.73 x 10^-7 M in veins and arteries, respectively. When evaluated by region, caudodorsal (n = 7) and cranioventral (n = 9) arteries did not differ in their responses to U 46619 (**Figure 9**, B)($p = 0.25$), while a regional effect was observed in pulmonary veins with cranioventral (n = 8) veins demonstrating enhanced sensitivity compared to caudodorsal veins (n = 7)(**Figure 9**, C)($p < 0.0001$).

Phenylephrine

Pulmonary arteries (n = 10) and pulmonary veins (n = 9) did not constrict in response to 10^-5 M phenylephrine (data not shown).

Isoproterenol

Isoproterenol caused a concentration-dependent relaxation in pulmonary arteries (n = 11), while pulmonary veins (n = 14) failed to respond. A significant difference between the responses of cranioventral (n = 5) and caudodorsal (n = 6) arteries to isoproterenol was detected ($p < 0.0001$)(**Figure 10**, A) with caudodorsal arteries demonstrating enhanced relaxation (relaxation 28.18 ± 3.74 % and 48.67 ± 3.09 % of maximum in CD and CV arteries respectively). There was no regional difference in the response of veins to isoproterenol (**Figure 10**, B).

Furosemide

Furosemide caused a mild, concentration-dependent relaxation in pulmonary arteries (n = 12)(relaxation 51.91 ± 24.31 % of maximum), and to a lesser degree, in pulmonary veins (n = 12)(**Figure 11**, A). DMSO vehicle did not cause relaxation in either pulmonary arteries (n = 5) or veins (n = 10)(**Figure 11**, B and C). Lung region did not affect responses of arteries or veins to furosemide ($p = 0.07$ and $p = 0.19$ for arteries and veins respectively)(**Figure 11**, B and C).

Methacholine

Methacholine caused a concentration-dependent relaxation in pulmonary veins (n = 13) but the response of arteries varied by region (n = 16). When arteries were evaluated by

region, all cranioventral arteries (n = 8) demonstrated a concentration-dependent relaxation in response to methacholine, while all caudodorsal arteries (n = 8) constricted, also in a concentration-dependent manner (**Figure12**, A). The response of pulmonary veins (n = 6 and n = 7 for CD and CV veins respectively) to methacholine was not affected by region (p = 0.59)(**Figure 12**, B).

Mechanisms of methacholine reactivity

Pre-incubation of caudodorsal pulmonary arteries (n = 7) with L-NAME did not affect their response (contraction) to methacholine (p = 0.7)(**Figure 13**, A). Pre-incubation with indomethacin however, augmented the contraction of CD arteries (n = 8) when compared to methacholine alone (p =< 0.0001)(Figure 5, B). Pre-incubation of CD arteries (n = 13) with both L-NAME and indomethacin also resulted in an augmented contraction (**Figure 13**, C) but the CCRC did not differ from vessels incubated with indomethacin alone (p = 0.79).

Pre-incubation of cranioventral pulmonary arteries (n = 8) with L-NAME diminished their response (relaxation) to methacholine (p < 0.0001)(**Figure 13**, D). Pre-incubation with indomethacin also resulted in reduced relaxation of CV arteries (n = 7)(**Figure 13**, E). Pre-incubation of CV arteries (n = 15) with both L-NAME and indomethacin induced a mild contraction and abolished relaxation until the highest concentrations of methacholine were added to the bath (1×10^{-6} and 3×10^{-6} M) (**Figure 13**, F). Bicarbonate buffer (used to make indomethacin stock solution) at the concentration used in the experiments did not affect PSS pH, making a vehicle effect improbable.

Endothelial imaging

Endothelium-covered regions were clearly distinguishable from denuded areas (**Figure 14**). Data from 8 CD and 11 CV vessels from the component of the study that investigated methacholine mechanisms are reported (remaining vessels from this study component could not be imaged). Values for percent endothelium-cover in a vessel were 43.84 ± 4.418 and 41.4 ± 3.617 (mean ± s.e.m.) for CD and CV vessels respectively, and these values did not differ between regions ($p = 0.8426$).

Histology

51 arteries (from both study components) and 21 veins were submitted for histologic evaluation. All vessels (with 3 exceptions) had their identity (as determined when they were dissected) confirmed by use of histology. 2 vessels that were dissected as veins were identified as arteries using histology, and data were included in the artery dataset. 1 artery could not be confirmed as such due to its orientation in the specimen processing gel, however based on a typical arterial reactivity profile, data from this vessel were also included in the study.

Discussion

The present study was designed to investigate whether regional differences in small pulmonary vessel reactivity exist in the horse, and whether detected differences could predict the distinct predilection of exercise-induced pulmonary hemorrhage (EIPH) lesions for caudodorsal lung (233). To the best of the authors' knowledge, this study reports for

the first time in any species, that regional differences in reactivity exist in small caliber pulmonary arteries and veins.

The vessels of interest in this investigation are small (100 – 400 μm O.D.) pulmonary arteries and veins. Evidence of regional heterogeneity in mechanical characteristics of equine pulmonary vessels of this caliber has recently been reported, and it is suggested that these differences are a reflection of the preferential distribution of blood flow in the equine lung to caudodorsal regions (196). Pulmonary veins in this size range from caudodorsal lung are remodeled in lungs of EIPH-affected horses, and are stiffer-walled in horses with a history of racing (196, 231, 233). Further study of these vessels was undertaken based on the following reasoning. The source of hemorrhage in EIPH is pulmonary capillary stress failure (230), resulting from elevated pulmonary circulation intravascular pressures in horses during exercise (124). Resistance to flow in the arteries and veins that supply and drain pulmonary capillaries determines capillary pressure. Therefore, regional arterial dilation resulting in transmission of high arterial pressures to the capillary bed, and/or venous constriction during exercise would increase capillary pressure and render capillaries in that region more susceptible to stress failure, and EIPH.

Before evaluation of vessel reactivity, a normalization procedure was performed in order to determine the degree of smooth muscle stretch that would result in maximal force development for subsequent experiments (7). Optimal passive wall tension of arteries was significantly larger than that of veins. Mean *in vivo* resting pulmonary arterial pressures are approximately 30 mmHg (106, 124, 190), while *in vivo* pulmonary artery wedge pressures (proxy for venous pressures) range from 13.4 to 18 mmHg(124, 128, 190). Conversion of optimal wall tension to equivalent pressure values demonstrated that these studies were

conducted at physiologically relevant tensions (24.2 and 14.1 mmHg in arteries and veins respectively).

The thromboxane A_2 analogue U 46619 was investigated with a view to its use in subsequent experiments in which pre-existing tone was necessary to study another vasoactive agent of interest. U 46619 caused contractions in both pulmonary arteries and veins, but pulmonary veins were more sensitive to U 46619 than arteries. While no regional differences in reactivity were observed in arteries, cranioventral veins were more sensitive than their caudodorsal counterparts. Thromboxane A_2 is an arachidonic acid metabolite derived from endothelial cells and platelets (147, 168) and enhanced sensitivity to U 46619 in pulmonary veins compared to pulmonary arteries has also been reported in other species, including sheep, dogs and guinea pigs (10, 99, 187). The role of thromboxane in regulation of vascular tone during exercise however, is thought to be minimal (144), and regional patterns of equine pulmonary vein sensitivity to this agent are unlikely to be significant in the context of EIPH.

Pulmonary arterial and venous tone is mediated, at least in part, by the autonomic nervous system, and during exercise, both sympathetic outflow and circulating concentrations of vasoactive catecholamines increase, while parasympathetic activity is diminished (133, 203). That this increase in sympathetic activity exerts an effect on the pulmonary circulation specifically during exercise is supported by data from both sheep and pigs (93, 203). For these reasons, investigations into how small pulmonary arteries and veins of the horse are affected by the autonomic nervous system during exercise, and whether regional heterogeneity in vessel reactivity exist were undertaken. Specifically, the selective α1- and non-selective β- adrenergic receptor agonists phenylephrine and

isoproterenol respectively, and the muscarinic agonist methacholine were studied. In the intensely exercising horse, circulating venous concentrations of epinephrine and norepinephrine increase from 0.9 and 0.7 to 153 and 148 nmol/L, respectively (191). These concentrations (approximately 1.5 x 10^-7 M) fall within the concentration range over which isoproterenol, an adrenergic agonist, was tested.

Even though large equine pulmonary arteries (1.5 – 4 mm in diameter)(117), and the largest pulmonary veins (63), contract in response to phenylephrine, indicating the presence of α1-receptors on the smooth muscle cells of these vessels, phenylephrine did not cause contraction in either arteries or veins in the present study, suggesting an absence of the target receptor in smaller caliber vessels. This observation is not unique to the horse. For example, small pulmonary arteries (100 – 300 μm in diameter) of the rat do not respond reliably to norepinephrine (108), and the response of ovine pulmonary arteries to norepinephrine is attenuated with decreasing vessel diameter (99).

With regard to the distribution of beta-adrenergic receptors, we found that pre-contracted equine pulmonary arteries relaxed in response to the non-specific β-adrenergic receptor agonist isoproterenol, while pulmonary veins failed to respond. Furthermore, isoproterenol-induced relaxation was of greater magnitude in the caudodorsal than cranioventral arteries (**Figure 10**). This is noteworthy because at this time, and to the best of the authors' knowledge, there are no other reports of regional differences in distribution of beta-adrenoreceptors in the pulmonary vasculature of any species.

In the small equine pulmonary vessels, β-adrenoreceptor-mediated vasodilation of small arteries will predominate during exercise (there was no evidence of α_1-adrenoreceptor-mediated constriction). A combination of a generalized failure of small

pulmonary veins to dilate, and enhanced dilation of caudodorsal pulmonary arteries in particular would expose capillaries in the caudodorsal lung to the greatest intravascular pressures during exercise, perhaps explaining, at least in part, the regional distribution of EIPH in the horse lung.

In this study, small, pre-contracted pulmonary veins of the horse relaxed in response to methacholine, and regional differences in the magnitude of this response were not detected. Pulmonary arteries on the other hand, demonstrated opposite effects depending on lung region. Arteries from caudodorsal lung contracted while those from cranioventral lung dilated in response to methacholine.

In general, binding of acetylcholine to muscarinic receptors on the endothelium, or on the smooth muscle of blood vessels, affects vessel tone by causing vasodilation and vasoconstriction respectively (133, 203). Whether equine blood vessels of this caliber are directly innervated by the parasympathetic nervous system has not been reported in detail, however, based on their responses to the acetylcholine analog methacholine, it is reasonable to infer that blood vessels studied in these experiments possess muscarinic receptors on the endothelium and/or smooth muscle. It is also considered unlikely that the regional differences in responses are explained by regional differences in endothelial coverage of vessels consequent to vessel injury during experimental manipulation. Percent endothelial cover in a subset of vessels in this study was determined based on the presence of the endothelial-specific CD31 antigen, and did not differ significantly between arteries from either lung region.

Regional differences in muscarinic-receptor mediated vessel reactivity have been reported before in the horse (164), and in the pig (176). However both studies report on

vessels that are much larger (4 – 6 mm O.D.) than those evaluated in this study. In porcine pulmonary arteries, more pronounced relaxation in response to acetylcholine was observed in dorsal vessels, compared to those from ventral lung (176), and large equine pulmonary arteries from caudodorsal lung also relax, while those from cranioventral lung contract in response to methacholine (164). The contrasting pattern observed in small equine pulmonary arteries is of particular interest when considered in the context of EIPH lesion distribution. If parasympathetic outflow is responsible for basal maintenance of tone in small pulmonary arteries in a region-dependent manner, perhaps fulfilling the role of protecting capillaries from high flow rates in this region, then diminished parasympathetic activity during exercise (133) could result in reduced arterial tone, in caudodorsal lung specifically. This, along with a possible attenuation of muscarinic-receptor mediated pulmonary venous dilation in the same region could result in transmission of higher pressures to caudodorsal pulmonary capillaries compared to capillaries in other lung regions. However, when extrapolating these data to *in vivo* conditions, it is noted that all vessels in these studies were pre-contracted with U 46619, and responses of pulmonary vessels to acetylcholine can vary depending on whether vascular tone is present (10).

Vasodilation associated with muscarinic-receptor activation is typically mediated by endothelium-derived nitric oxide (NO) and/or prostanoids, commonly PGI_2; whereas vasoconstriction can result from generation of vasoconstrictor prostanoids termed endothelial derived constricting factors (EDCFs) (236) and/or direct binding of vascular smooth muscle muscarinic receptors (133). In order to further investigate the role of nitric oxide and prostanoids in muscarinic-receptor mediated equine pulmonary artery vasomotion, a subset of small arteries were incubated with L-NAME, a nitric oxide synthase

inhibitor and/or indomethacin, a cyclooxygenase inhibitor, before treatment with methacholine.

L-NAME did not affect the response of caudodorsal vessels to methacholine, while indomethacin pre-incubation resulted in augmented contraction. These data indicate that some prostanoid-mediated dilation was occurring in caudodorsal arteries, but was masked by the magnitude of the contraction. L-NAME caused attenuation of vasodilation in cranioventral arteries, as did indomethacin. Co-incubation with both inhibitors prevented any relaxation of cranioventral vessels until the highest methacholine concentrations were applied. These data implicate roles for both nitric oxide and prostanoids in cranioventral artery relaxation.

That nitric oxide contributes to maintenance of basal pulmonary vasomotor tone has been demonstrated in the horse. Supplemental nitric oxide administration causes a significant decrease in mean peak pulmonary artery pressure in exercising horses, suggesting that the pulmonary vasculature is not fully dilated during exercise (100, 135). Furthermore, administration of L-NAME to horses at rest results in significant increases in pulmonary arterial, capillary and venous pressures (126). Our data demonstrate that nitric oxide could play a role in small pulmonary artery vasomotion *in vivo*, at least in the cranioventral lung, but the effect of these specific arteries on whole lung vascular pressure data is not known.

Cyclooxygenase inhibitors are commonly used to treat musculoskeletal abnormalities in performance horses (137). The effect of cyclooxygenase inhibition on pulmonary vasculature as an off-target effect of non-steroidal anti-inflammatory medications may merit future consideration.

The loop diuretic furosemide is commonly administered to horses before racing to control EIPH (193), and has been demonstrated to reduce both the severity and the incidence of EIPH in Thoroughbreds (74). This protective effect of furosemide is commonly attributed to a decrease in mean pulmonary artery pressure during strenuous exercise (71, 129) as a result of a diuresis-associated reduction in plasma volume (71). However, furosemide administration to horses results in redistribution of pulmonary blood flow without a concomitant drop in cardiac output (43). This information suggests that furosemide also affects equine pulmonary vascular reactivity.

Furosemide is a pulmonary venodilator in the dog, but does not dilate pre-contracted pulmonary arteries (59). Therefore we investigated the hypothesis that furosemide would dilate pulmonary veins but not pulmonary arteries independent of lung region. Contrary to our hypothesis, pre-contracted pulmonary arteries relaxed in response to high concentrations of furosemide in a non-region dependent manner, and mild relaxation was also observed in veins from both lung regions. The clinical relevance of this finding must be considered negligible as the concentration at which maximal effects were observed in the present study is 1000 times higher than plasma levels of furosemide in horses one hour after intravenous administration of 1 mg/kg (24).

It is worth noting however, that the pulmonary veins from dogs in which a dilator effect was seen were larger (1 – 1.2 mm O.D.) than vessels in the present investigation(59). Significant differences in reactivity to various pharmacologic agents between vessels of different size have been reported in many species including rats (108), pigs (239) and sheep (99). For this reason, the effect of furosemide on equine pulmonary veins of a larger caliber is considered worthy of future investigation.

Data in this study were acquired from horses that had not raced. Horses that have trained and raced have remodeled pulmonary veins (233), and racing is associated with increased wall stiffness of caudodorsal veins (196). It is reported in pigs that remodeling of pulmonary vessels and associated structural changes are associated with alterations in vessel reactivity (98). Therefore, remodeled pulmonary veins such as those seen in EIPH-affected lung (231) may react differently than vessels used in this study. Furthermore, exercise training has been demonstrated to improve pulmonary artery dilation in response to acetylcholine (87) in pigs. Future investigations into whether there is an effect of exercise and associated remodeling on equine pulmonary vascular reactivity may shed further light on progression of EIPH over the course of a horse's athletic career.

APPENDIX

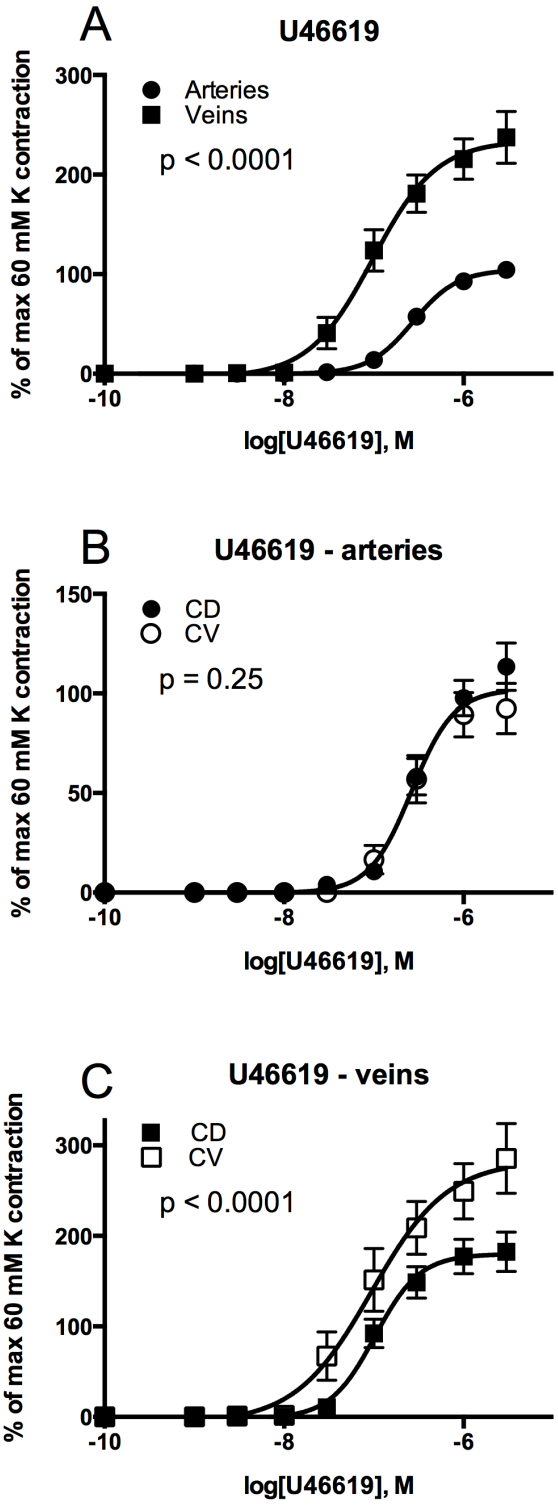

Figure 9 Cumulative concentration response curves for U46619 for all arteries and veins (*A*), caudodorsal (CD) and cranioventral (CV) arteries (*B*), and CD and CV veins (*C*). Values

Figure 9 (cont'd) are means ± SE. Veins are more sensitive to U 46619 than arteries (A); regional differences in responses of CD and CV arteries do not exist ($p = 0.25$)(B) whereas CV veins are more sensitive to U 46619 than CD veins ($p < 0.0001$)(C).

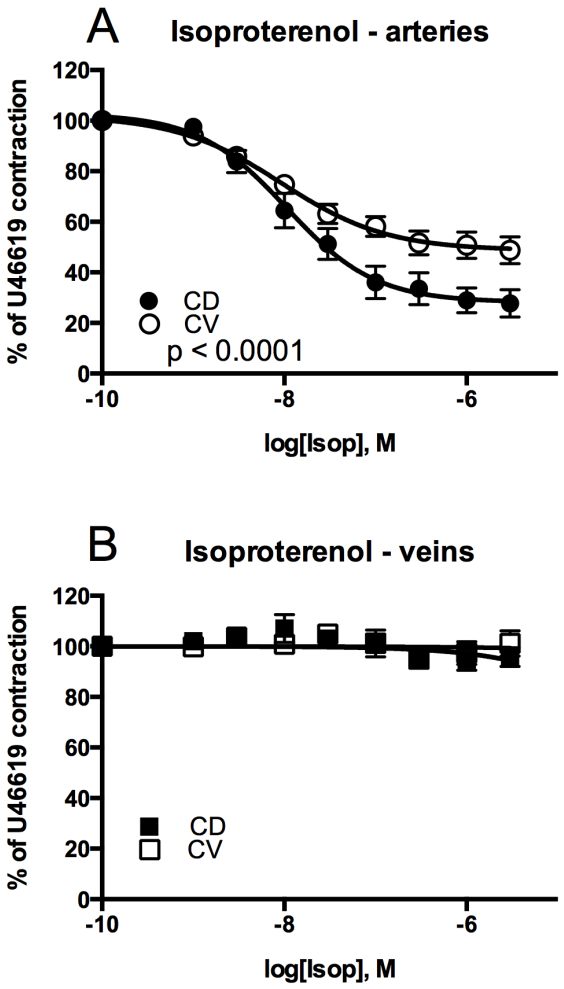

Figure 10 Cumulative concentration response curves for isoproterenol for caudodorsal (CD) and cranioventral (CV) arteries (*A*), and CD and CV veins (*B*). Values are means ± SE. Concentration-dependent relaxation is greater in CD compared to CV arteries ($p < 0.0001$)(*A*), whereas pre-contracted veins do not relax in response to isoproterenol, in both CD and CV regions (*B*).

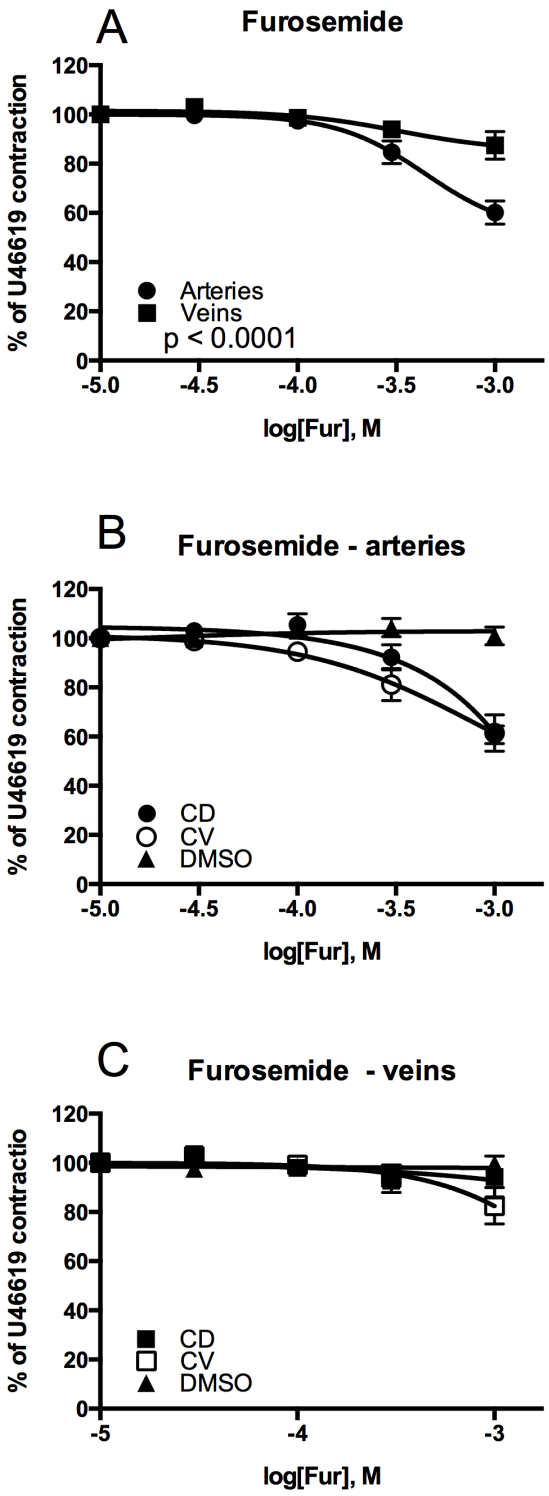

Figure 11 Cumulative concentration response curves for furosemide for all arteries and veins (*A*), caudodorsal (CD) and cranioventral (CV) arteries (*B*), and CD and CV veins (*C*). Values are means ± SE. Mild concentration-dependent relaxation to furosemide occurs in

Figure 11 (cont'd) arteries, and to a lesser degree in veins (*A*); regional differences in the response of arteries and veins to furosemide do not exist ($p = 0.07$ and $p = 0.19$ for arteries and veins respectively)(*B* and *C* respectively). DMSO vehicle (dark triangle) does not affect arteries and veins (*B* and *C* respectively).

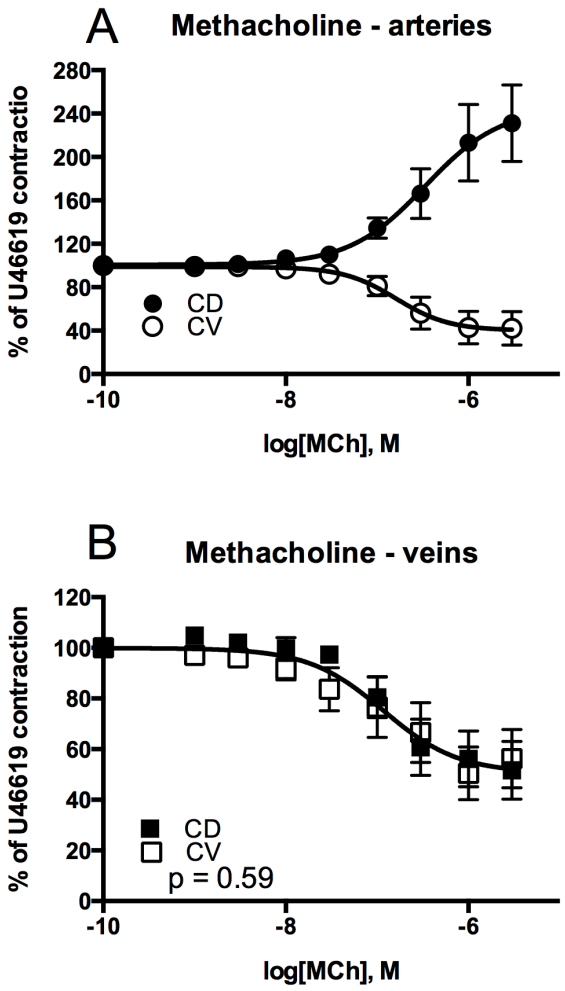

Figure 12: Cumulative concentration response curves for methacholine for caudodorsal (CD) and cranioventral (CV) arteries (*A*), and CD and CV veins (*B*). Values are means ± SE. Concentration-dependent relaxation occurs in CV arteries, and concentration-dependent constriction occurred in CD arteries (*A*); pre-contracted pulmonary veins relax in a concentration-dependent manner, regardless of region (*B*).

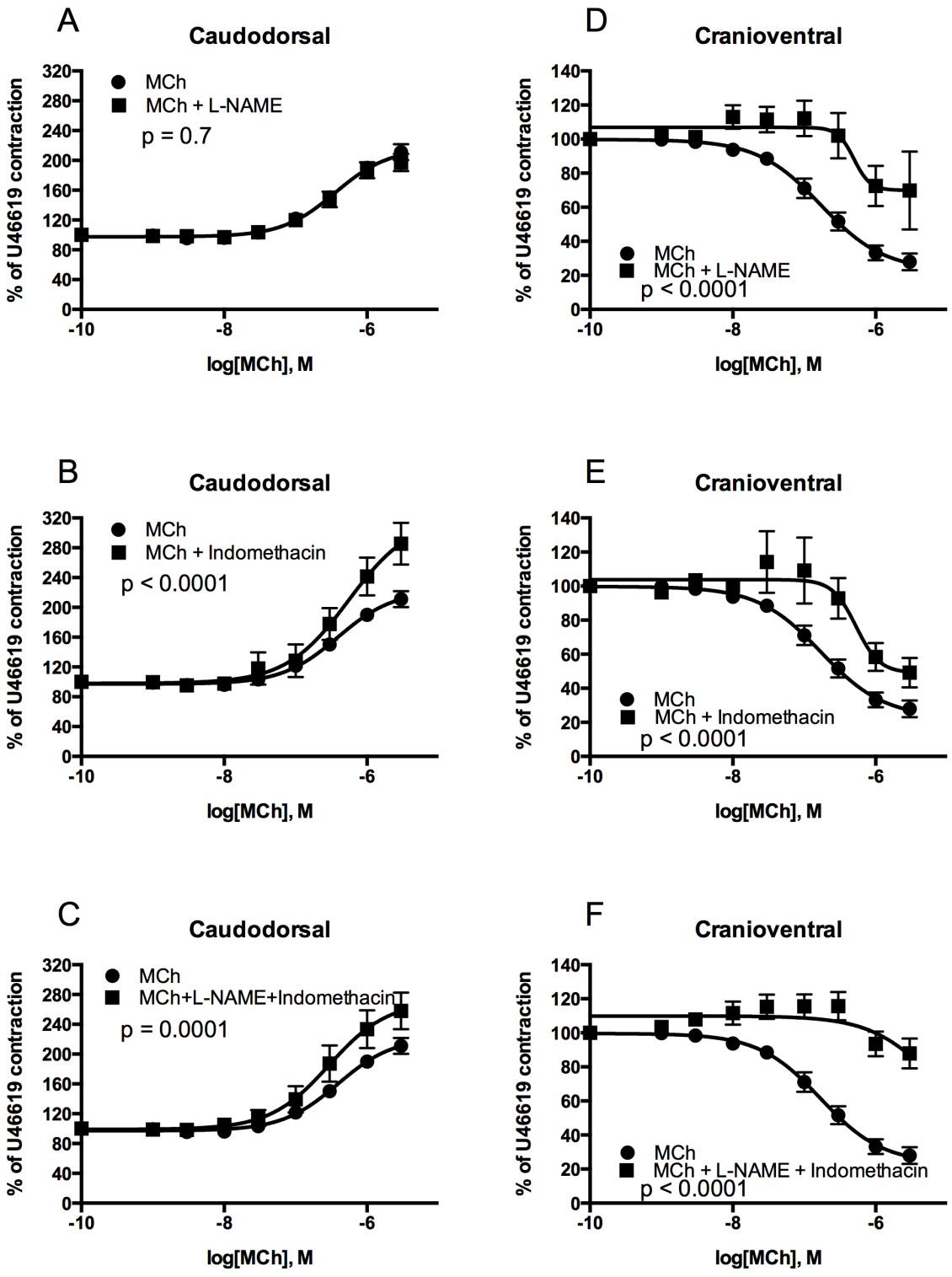

Figure 13: Cumulative concentration response curves for CD (*A, B,* C) and CV (*D, E, F*) arteries comparing responses to methacholine (MCh) only, with responses to MCh applied

Figure 13 (cont'd) after pre-incubation with L-NAME (*A* and *D*), indomethacin (*B* and *E*), and L-NAME and indomethacin (*C* and *F*). Values are means ± SE. Pre-incubation with L-NAME does not affect CD artery constriction in response to MCh (*A*) whereas CV artery relaxation is partially inhibited by L-NAME (*D*). Indomethacin pre-incubation augments CD artery constriction, and partially inhibits CV artery relaxation (*B* and *E* respectively). Pre-incubation with both L-NAME and indomethacin caused enhanced MCh-induced constriction in CD arteries (*C*) and a mild contraction followed by mild relaxation in CV arteries (*D*).

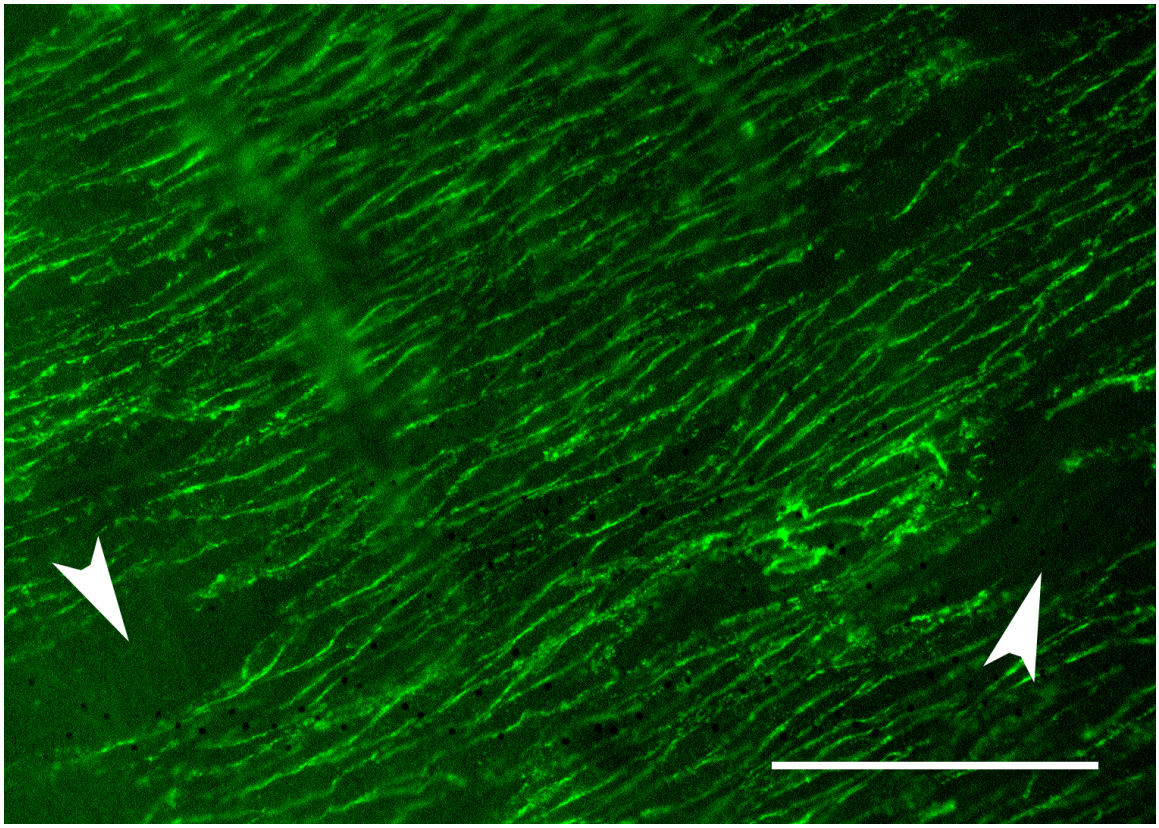

Figure 14 Fluorescent staining of CD-31 on endothelial surface of equine pulmonary artery. Regions of intact endothelium can be discerned from endothelium-denuded, non-stained regions (indicated by arrow-heads). Scale bar = 100 μm.

CHAPTER 4

Effects of exercise on markers of venous remodeling in lungs of horses

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Am J Vet Res. 2013 Sep;74(9):1231-8. doi: 10.2460/ajvr.74.9.1231.

Abstract

Objective: To determine the effects of 2 weeks of intense exercise on expression of markers of pulmonary venous remodeling in caudodorsal and cranioventral regions of lungs of horses.

Animals: 6 horses.

Procedures: Tissue samples of caudodorsal and cranioventral regions of lungs were obtained before and after conditioning and 2 weeks of intense exercise. Pulmonary veins were isolated and assayed via quantitative real-time PCR to determine mRNA expression of matrix metalloproteinase-2 and -9, tissue inhibitor of metalloproteinase-1 and -2, collagen type I, tenascin-C, endothelin-1, platelet derived growth factor, transforming growth factor-β (TGF-β), and vascular endothelial growth factor (VEGF). Protein expression of collagen (via morphometric analysis) and tenascin-C, TGF-β, and VEGF (via immunohistochemistry) was determined.

Results: Exercise-induced pulmonary hemorrhage was detected in 33.3% of horses after exercise. The mRNA expression of matrix metalloproteinase-2 and -9, tissue inhibitor of metalloproteinase-2, TGF-β, and VEGF was significantly lower in pulmonary veins obtained

after exercise versus those obtained before exercise for both caudodorsal and cranioventral regions of lungs. Collagen content was significantly higher in tissue samples obtained from caudodorsal regions of lungs versus those obtained from cranioventral regions of lungs both before and after exercise. Exercise did not alter protein expression of tenascin-C, TGF-β, or VEGF.

Conclusions and Clinical Relevance: Results of this study indicated 2 weeks of intense exercise did not alter expression of marker genes in a manner expected to favor venous remodeling. Pulmonary venous remodeling is complex and more than 2 weeks of intense exercise may be required to induce such remodeling.

Introduction

Exercise-induced pulmonary hemorrhage is common in racehorses after intense exercise; EIPH is detected in up to 75% of such horses via endoscopic evaluation of respiratory tracts (162, 172). Horses with no or very mild EIPH are four times as likely to win a race as horses with moderate or severe EIPH (70), suggesting this condition has negative effects on racehorse performance.

The predominant location of EIPH lesions in horses is the caudodorsal regions of lungs (150, 157, 231). A distinctive histopathologic lesion of EIPH is remodeling of small-diameter pulmonary veins (venous remodeling) (231). Venous remodeling is characterized by collagen deposition in walls and smooth muscle hypertrophy of veins resulting in thickening of walls and narrowing of lumens (38). Other lesions of EIPH include pulmonary

interstitial and septal fibrosis, hemosiderin accumulation in lung tissue, and bronchial circulation neovascularization(38, 152).

Pulmonary venous remodeling has potentially important physiologic effects on vascular pressures in lungs. During exercise, horses have a substantial increase in pulmonary intravascular pressures (106, 127). Estimated pulmonary capillary pressures in horses are between 17.8 mmHg (190) to 25 mmHg (127) at rest and 72.5 mmHg (106) to 83.3 mmHg (127) during exercise. Such transmural pulmonary capillary pressures can cause blood vessel rupture and EIPH (17). A decrease in the lumen size of pulmonary veins would further increase pulmonary capillary pressures. Complete pulmonary venous occlusion would cause capillary pressures equal to pulmonary arterial pressures, which can be > 96.5 mmHg (106, 127). Remodeling of systemic (in rabbits, rodents, and pigs)(2, 28, 29, 64, 112, 235) and pulmonary (in humans and sheep)(25, 86)veins can develop when such blood vessels are exposed to high intravascular pressures.

Results of studies of vasculature in humans (146, 163, 222), pigs,(29, 224) and rodents(225, 234) indicate venous remodeling is preceded by alterations in mRNA expression of proteins that are important in the remodeling process. These proteins include MMPs, TIMPs (29, 225), collagen (234), tenascin-C (222), and various growth factors that are produced by fibroblasts in vein walls and monocytes and macrophages (146, 163, 199, 224, 234). The objective of the study reported here was to determine mRNA expression of MMP-2, MMP-9, TIMP-1, TIMP-2, collagen type I, tenascin-C, ET-1, PDGF, TGF-β, and VEGF and protein expression of tenascin-C, TGF-β, and VEGF in pulmonary veins obtained from caudodorsal and cranioventral regions of lungs of horses before and after 2 weeks of intense exercise. Because the amount of collagen in lung parenchyma of

horses with EIPH is greater than that for horses without EIPH (primarily in the caudodorsal regions of lungs(38)), we also compared collagen content in parenchyma of caudodorsal and cranioventral regions of lungs of horses before and after 2 weeks of intense exercise. The hypothesis was that 2 weeks of intense exercise would alter mRNA and protein expression of the evaluated factors in pulmonary veins of caudodorsal but not cranioventral regions of lungs of horses in a manner expected to favor vascular remodeling. In addition, we hypothesized that exercise of horses would cause an increase in the collagen content of caudodorsal but not cranioventral regions of lungs.**9**

Materials and Methods

Animals

Seven horses (six geldings and one sexually intact female; age range, 2 to 4 years; body weight range, 350 to 473 kg) of non-racing breeds were purchased for use in this study. These horses had not been previously trained for any purpose and were selected for inclusion in the study because it was unlikely that they had prior EIPH episodes. Horses were not vigorously exercised for at least two months before the study. The horses were determined to be healthy on the basis of results of physical examinations and tracheobronchoendoscopy. One horse was excluded from the study because of lameness. Therefore, the study was completed and data were analyzed for 6 horses. The Michigan State University Institutional Animal Care and Use Committee approved this study.

Experimental protocol

Pulmonary wedge resections were performed via a thoracoscopic technique for standing horses. Before undergoing an intense exercise protocol, lung samples were obtained from cranioventral and caudodorsal regions of left or right lungs (determined via a randomization procedure) of each horse. Horses were then returned to pasture for at least 6 months. Subsequently, horses underwent conditioning and intense exercise during a 4-week period. After completion of the intense exercise protocol (first exercise period), pulmonary wedge resections were performed to obtain lung samples from cranioventral and caudodorsal regions of right or left of horses (lung contralateral to the lung from which samples were obtained before exercise); the mRNA prepared from these lung samples was of poor quality and low quantity. Therefore, horses were rested for a further 6 months and the exercise protocol was repeated (second exercise period). Subsequently, tissue samples from the cranioventral and caudodorsal regions of the same lung (contralateral to the lung from which samples were obtained before the first exercise protocol) were collected during general anesthesia of horses. Lung samples were obtained from sites that had not previously undergone surgery. A long time was allowed between pulmonary wedge resection procedures to minimize the effects of previous surgeries on gene expression.

Exercise protocol

Horses underwent a 2-week period of conditioning followed by a 2-week period of intense exercise intended to simulate race training. Horses were conditioned 5 days/week for 2 weeks on a high-speed treadmill with a 0% incline. After two weeks of conditioning, the HR_{max} of each horse was determined via a rapid incremental exercise test (206). Briefly, heart rates were determined by use of a telemetric system; the HR_{max} was determined to be

the heart rate at which an increase in treadmill speed did not result in an increase in HR. The treadmill speed corresponding to 120% of HR_{max} was determined via extrapolation.

After the 2-week conditioning period horses were intensely exercised on 6 days (intense exercise days 1, 3, 5, 8, 10 and 12). Each exercise session included a 4-minute warm-up period followed by exercise at a treadmill speed corresponding to 120% of HR_{max} for 2 minutes or until the horse could no longer maintain its position on the treadmill. Within 45 to 90 minutes after the end of the final exercise session of the first exercise period, horses underwent endoscopic examination of the trachea. Endoscopic examination of horses was not repeated after the second exercise period because the intensity of exercise during the first period was determined to have been adequate to induce EIPH. An established grading system (grade 0 = no blood visible in trachea; grade 4 = >90% of tracheal surface covered in blood)(69) was used to determine EIPH severity in study horses.

Pulmonary wedge resection

During each pulmonary wedge resection procedure, 2 lung samples were obtained from each horse (one each from the cranioventral and caudodorsal regions of the left or right lung) (115). Briefly, each horse was restrained in stocks and sedated with a continuous IV infusion of detomidine hydrochloride (initial dose of 6 μg/kg followed by 0.8 μg/kg/min). Mepivacaine (20 to 30 ml of a 2% solution) was injected SC and in intercostal muscles at each surgery site. Intercostal nerves at surgery sites were blocked at the level of vertebral transverse processes with 0.75% bupivacaine (5 mL/site). Antimicrobial drugs (penicillin G potassium [22,000 IU/kg, IV, q 6 h] and gentamicin sulfate [6.6 mg/kg, IV, q 24h]) and an

NSAID (flunixin meglumine [1.1 mg/kg, IV, q 12 h]) were administered during surgery after lung samples had been obtained (to avoid potential effects of drugs on gene expression).

For thoracoscopy, a 30-degree rigid endoscope (10 mm x 58 cm)(Hopkins telescope, Karl Storz Veterinary Endoscopy, Goleta, CA.), video camera (Vetcam, Karl Storz Veterinary Endoscopy, Goleta, CA), light cable, and 250-watt xenon light source (Stryker Quantum 3000, Stryker Endoscopy, Kalamazoo, MI) were used. Pneumothorax was induced and lungs were deflated via insertion of a teat cannula into the pleural space.

Six instrument portals were made in the thoracic wall (3 for each lung sample collection site [one each for an endoscope, forceps, and stapler]). The caudodorsal lung sample collection site was accessed via intercostal spaces 12, 13 and 15; the cranioventral site was accessed via intercostal spaces 7 and 8. Endoscopic atraumatic forceps (10 mm atraumatic Babcock forceps, Ethicon Endo-Surgery Inc, Cincinnati, OH) were used to manipulate lungs.

An endoscopic stapler (ETS45 Endoscopic linear cutter, Ethicon Endo-Surgery Inc, Cincinnati, OH) was used to perform pulmonary wedge resections. Lung samples (approx 4 cm long) were obtained from each site. Lungs were reinflated by withdrawing air from the thorax and skin at portal sites was closed with sutures in a simple interrupted pattern. Antimicrobial and NSAID administration was continued for 7 days after surgery.

Pulmonary wedge resections were performed within 24 hours after completion of the first exercise period to collect lung samples from the lung contralateral to the lung from which tissue samples had been obtained before exercise. Within 24 hours after completion of the second exercise period, each horse was anesthetized (xylazine hydrochloride [1.1 mg/kg, IV] followed by ketamine hydrochloride [2.2mg/kg, IV]) and placed in left or right

lateral recumbency. Lung samples were obtained via thoracotomy and previous surgery sites were avoided. Immediately after lung samples were obtained, anesthetized horses were euthanized with pentobarbital sodium (90mg/kg, IV).

Harvesting of pulmonary veins

Immediately after collection, lung samples were divided into 2 approximately equal pieces; one was placed in a storage solution (RNAlater, Ambion, Life Technologies, Carlsbad CA) and kept at 4°C for 24 hours, and then stored until use at - 20°C. The other piece of each lung sample was fixed in 10% neutral buffered formalin and embedded in paraffin for histologic examination and morphometric and immunohistochemical analyses; 6 µm-thick sections of lung tissue were placed on glass slides and stained with H&E, picrosirius red, and Verhoeff-Van Gieson stains.

For lung samples in storage solution (RNAlater), intralobular pulmonary veins (length, 0.5 to 3 mm) were collected by use of a dissecting microscope (Olympus SZX16, Olympus America Inc, Center Valley, PA). During preliminary studies, accurate identification and dissection of pulmonary veins from peripheral lung tissue had been validated via histologic techniques. For each horse, all veins harvested for each lung collection site and time were pooled for mRNA extraction.

mRNA extraction

Pulmonary vein samples were removed from storage solution[f] and placed in 400 µL of lysis buffer (Buffer RLT, Qiagen Inc, Valencia, CA)(containing β-mercaptoethanol). Pulmonary vein samples were processed with a tissue grinder (Kontes Glass Co Duall 21, Fischer

Scientific, Pittsburgh, PA). Total RNA was extracted with a kit (RNeasy Micro Kit, Qiagen Inc, Valencia, CA) and a homogenizer (QIAshredder, Qiagen Inc, Valencia, CA); DNase digestion (RNase-Free DNase Set, Qiagen Inc, Valencia, CA) was used in conjunction with RNA extraction in an attempt to remove genomic DNA.

The purity and concentration of RNA in each sample were determined with a spectrophotometer (NanoDrop 1000 Spectrophotometer, NanoDrop Products, Wilimington, DE). In addition, RNA integrity number (183) was determined by use of a bioanalzyer system (2100 Bioanalyzer with RNA Pico 6000 kit, Aligent Technologies, Santa Clara, CA). To ensure adequate purity and concentration of mRNA, only samples with 260 nm-to-280 nm absorbance ratios between 1.9 and 2.2 were used. In addition, only mRNA samples with an RNA Integrity Number > 5 were used (47).

As a result of these criteria, all samples obtained after the first exercise period and samples obtained from 2 horses after the second period were not assayed. Therefore, mRNA samples for 4 horses prepared from lung samples obtained after the second exercise period were assayed. Both horses with EIPH (endoscopic diagnosis) were included in the final analysis.

Then, cDNA was synthesized (High Capacity cDNA Reverse Transcription Kit with RANse inhibitor, Applied Biosystems, Life Technologies, Carlsbad, CA) and amplified (TaqMan PreAmp Master Mix, Applied Biosystems, Life Technologies, Carlsbad, CA) because of low cDNA concentrations.

Quantitative real-time PCR assays

The qRT-PCR assays were performed with a PCR system (7500 Fast Real-Time PCR system, Biosystems, Life Technologies, Carlsbad, CA) operating in standard mode with custom-designed probes (**Table 2**)(Informatics pipeline software, Applied Biosystems, Life Technologies, Carlsbad, CA).

The primer design variables for each gene were tested extensively, resulting in 100% PCR efficiency of a 6-log dilution range for mRNA samples free of PCR inhibitors. The qRT-PCR reactions were performed in triplicate with a 20 μL reaction mixture for each reaction well; reaction mixtures contained 10 μL of a DNA polymerase and dNTP mixture (TaqMan Gene Expression Master Mix, Applied Biosystems, Life Technologies, Carlsbad, CA), 1 μL of a mixture of forward and reverse primers and custom-designed probes (Custom TaqMan Gene Expression Assay Mix, Applied Biosystems, Life Technologies, Carlsbad, CA), 5 μL of amplified cDNA, and 4 μL of nuclease-free water. Expression of MMP-2, MMP-9, TIMP-1, TIMP-2, collagen type I, tenascin-C, ET-1, PDGF, TGF-β and VEGF were determined via qRT-PCR assays.

The qRT-PCR assays were performed at 50^{o} C for 2 minutes, 95^{o} C for 10 minutes, and 40 cycles of 95^{o} C for 15 seconds and 60^{o} C for 1 minute. The endogenous control values for the RT-PCR assay were mean values of beta-actin, beta-2-microglobulin, and elongation factor-1alpha expression.

Fold changes in gene expression were calculated via the $2^{-\Delta\Delta CT}$ method (113). Statistical analyses were performed with ΔCT values; for each mRNA sample and gene of interest, the ΔCT values were defined as the mean CT (cycle threshold) value of the gene in a sample minus the mean CT value of the control genes in that same sample.

Immunohistochemistry

The 6 μm-thick lung tissue sections were deparaffinized in xylene and rehydrated in a graded series of concentrations of ethanol. Lung sections were incubated overnight at 4°C with antibodies against tenascin-C (1:100) (Tenascin-C (BC-24): sc-59884, SantaCruz Biotechnology, Santa Cruz, CA), TGF-β (1:100) (TGF-β (V): sc-146, and blocking peptide,), or VEGF (1:100)(VEGF (147): sc-507, SantaCruz Biotechnology, Santa Cruz CA).

To ensure antibody-binding specificity, a peptide blocking (sc-146 and sc-507 blocking peptides, SantaCruz Biotechnology, Santa Cruz, CA) step was used for antibodies against TGF-β and VEGF, and nonspecific rabbit IgG was used for antibodies against tenascin-C. For each antibody, an appropriate positive control tissue was analyzed.

Following incubation with primary antibodies, lung sections were incubated with rabbit (TGF-β and VEGF) or mouse (tenascin-C) biotinylated secondary antibody. Then, slides were incubated with avidin-biotin conjugated horseradish peroxidase (Vectastain Elite ABC System, Vector Laboratories Inc, Burlingame, CA) and antibodies were detected with a peroxidase substrate (NovaRED Peroxidase Substrate Kit, Vector Laboratories Inc, Burlingame, CA).

A board-certified veterinary pathologist (KJW) who was unaware of the exercise status of horses and sample locations of lung tissue sections evaluated all slides via bright field microscopy. Pulmonary vein protein expression was scored as 0 (no evidence of protein expression), 1 (mild protein expression in a small number of veins), or 2 (strong protein expression in most [> 50%] veins).

Collagen content analysis

Picrosirius red staining and polarized microscopy of tissue samples is commonly used for detection and quantification of collagen (38, 169). For the quantification of collagen in lung tissue samples in the present study, picrosirius red–stained slides were scanned and digitalized at a magnification of 20X with a virtual slide system (VS120-SL, Olympus America Inc, Center Valley, PA). Polarization filters were used to enhance the appearance of the picrosirius red stain in images of lung tissue samples.

Automated random subsampling was performed on each of the digitalized slides with stereology software (NewCAST whole slide stereology software, Visiopharm, Hoersholm, Denmark)(magnification, 20X), and 50 images per slide were analyzed. Some lung sample slides had pleural tissue; such regions were excluded from analysis.

Morphological determination of the percentage of collagen in lung tissue samples was performed with software (http://www.stepanizer.com) (211). Briefly, a point grid with a density of 7 X 7 points/98,157 μm^2 was superimposed over images and all points that contacted noncollagenous lung tissue and those that contacted collagenous tissue were counted. The percentage of collagen in lung tissue samples was estimated by dividing the number of points that contacted collagen by the total number of points counted.

Statistical analyses

The ΔCT values were evaluated for normality and transformed as needed for statistical analysis.

The resulting data were analyzed with the following model (PROC MIXED, SAS Institute Inc, Cary, NC) to determine effect of exercise on the expression of each gene:

$$Y_{ijkl} = \mu + Site_k + Status_l + Site \times Status + Horse_i + e_{ijkl}$$

where Y_{ijkl} is the normalized gene expression of a gene of interest for horse $_i$ in sample $_j$ that corresponds to lung site $_k$ (caudodorsal or cranioventral) and status $_l$ (before or after exercise); μ is the mean value for the population; and e_{ij} is the residual. Horse effects were assumed to be random to account for within horse measurement correlations; residuals within each horse were heteroskedastic for lung samples obtained before and after exercise, indicating there were different variances for those groups. This model is practically equivalent to using a joint mixed model analysis of test and control genes (198).

For immunohistochemistry data, the Wilcoxon Signed-Rank Test (NCSS Statistical Software, Kaysvill, UT) for nonparametric data was used for analyses. The pre- and postexercise scores were compared for each lung sample collection site.

For collagen content data, a 3-factor (2-factor repeated measures) analysis of variance (PROC MIXED, SAS Institute Inc, Cary, NC) was used for analyses with site (caudodorsal or cranioventral) and time (before or after exercise) as fixed factors and horse as the random factor. Bonferroni's correction for multiple comparisons was used. A normal distribution of errors was determined via the Shapiro-Wilks' test. Collagen content data were reported as least square means ± SEM.

Values of $P < 0.05$ were considered significant.

Results

Two of 6 horses that finished the study had tracheobronchoscopic evidence of pulmonary hemorrhage within 90 minutes after the end of the final high-intensity exercise session

during the first exercise period; therefore, 33.3% of horses had EIPH at that time. The EIPH severity grade for both of those horses was 1 of 4 (69).

The mRNA prepared from pulmonary vein samples were of insufficient quality for analysis for all horses after the first exercise period and for 2 horses after the second exercise period; therefore, mRNA samples for 4 horses obtained after the second exercise period were analyzed via PCR assay for determination of gene expression. Results of initial analysis indicated exercise of horses had an effect on gene expression in pulmonary vein samples, but the interaction of the variables pulmonary wedge resection site (caudodorsal vs cranioventral) X exercise was not significant. Therefore, mean values for gene expression in pulmonary vein samples obtained from the caudodorsal regions of lungs and for those obtained from the cranioventral regions of lungs were used for analysis. Exercise of horses significantly decreased expression of 5 of the 10 genes evaluated (MMP-2 [P = 0.017], MMP-9 [P = 0.035], TIMP-2 [P = .039], TGF-β [P = 0.003], and VEGF [P = 0.007]; (**Figure 15**). Gene expression did not significantly change after exercise for TIMP-1 (P = 0.270), collagen type I (P = 0.130), tenascin-C (P = 0.659), ET-1 (P = 0.077), and PDGF (P = 0.119).

The only gene with differential expression between pulmonary vein samples obtained from caudodorsal regions of lungs and those obtained from cranioventral regions of lungs was tenascin-C. The mRNA expression of tenascin-C was approximately four times as greater in pulmonary vein samples obtained from cranioventral regions of lungs as it was in samples obtained from caudodorsal regions of lungs; these gene expression values were significantly (P = 0.033) different. However, tenascin-C expression in each of those lung regions did not significantly change after exercise.

Protein expression in lung samples was determined via immunohistochemical methods for all 6 horses that completed the study; results indicated exercise had no effect on protein expression of tenascin-C, TGF-β, or VEGF in tissue samples obtained from caudodorsal or cranioventral regions of lungs. The percentage of collagen in tissue samples obtained from caudodorsal regions of lungs was significantly (P <0.05) higher than that in tissue samples obtained from cranioventral regions of lungs, although the percentage of collagen was not significantly different in lung samples obtained before and after exercise **(Figure 16)**.

Discussion

Venous remodeling is important in the pathogenesis of EIPH. Alterations in mRNA expression are expected to precede structural changes in vasculature. Therefore, the purpose of this study was to determine whether 2 weeks of intense exercise would affect mRNA and protein expression of mediators of pulmonary intralobular vein remodeling in a manner expected to favor vascular remodeling in caudodorsal but not cranioventral regions of lungs of horses.

The thoracoscopic technique used to obtain lung samples from standing horses in this study was previously reported (115) and validated by personnel in our laboratory. No intraoperative complications were detected, and horses had no substantial problems attributable to the surgery. The endoscopic device used to obtain lung samples resulted in collection of an adequate amount of tissue for harvest of veins and preparation of mRNA. To reduce the effects of surrounding tissues on results for pulmonary veins, a microdissection technique was used to ensure that only the cells of interest (intralobular venous wall cells) were isolated and assayed.

The markers of venous remodeling evaluated in the present study were selected on the basis of studies conducted with animals of other species, because such information was not available for horses, to the authors' knowledge. The activities of MMP-2 and MMP-9, which have predominantly proteolytic actions, are regulated by TIMP-1 and TIMP-2 (142); these factors regulate the protein content of extracellular matrix. In general, hypertension results in increased expression of MMP-2 and MMP-9 mRNA or protein (109, 225) and decreased (29, 235) or no change (23) in TIMP expression.

Results of other studies indicate collagen content is increased in severely affected regions of lungs of horses with EIPH (38) and in walls of remodeled veins in humans (25) and rabbits (235). Tenascin-C (an extracellular matrix protein) expression is upregulated by MMPs (89) and PDGF (223) and is expressed during venous remodeling (2, 222). Endothelin-1 causes vasoconstriction in vivo (136) and has been implicated in pulmonary (204) and systemic (224) venous remodeling. Platelet-derived growth factor is a potent mitogen of connective tissue cells (65) and is associated with venous remodeling in pigs (48). The cytokine TGF-β is important in various developmental and pathological processes (161) and has been implicated in vein graft remodeling (85). Vascular endothelial growth factor is also a mitogen that is produced by vascular endothelial cells(46); that cytokine has a role in formation of neointima in remodeled blood vessels (156, 241).

We expected that expression of the genes evaluated in this study (except TIMPs) would increase in pulmonary veins of caudodorsal regions of lungs after exercise of horses. Results of this study indicated that mean expression values of all genes evaluated decreased in pulmonary veins after exercise; these findings were significant for MMP-2, MMP-9, TIMP-2, TGF-β, and VEGF. Because the collection site X treatment interaction was

not significant, decreases in expression were attributed to causes other than lung region. Although expression of tenascin-C mRNA was not increased after exercise, tenascin-C mRNA expression was higher in pulmonary veins in cranioventral regions of lungs versus those in caudodorsal regions of lungs.

The main advantage of qRT-PCR assays for determination of gene expression in pulmonary veins is that the technique has high sensitivity; therefore, mRNA expression can be determined for small amounts of tissue. Furthermore, expression of multiple genes can be evaluated for a tissue sample via that technique. Data regarding expression of mRNA are commonly used to infer other information about molecular pathways in cells, including information regarding protein expression. However, because of translational and posttranslational control mechanisms, such inferences may not be correct (186). For example, results of another study indicate differential mRNA and protein expression of MMP-2, MMP-9, and TIMP-1 (111). Because of this possibility, we determined vascular expression of TGF-β, VEGF, and tenascin-C via immunohistochemical methods. Unlike the results for gene expression, no significant decrease in protein expression was detected by use of that semiquantitative method in the present study. Immunohistochemistry was used rather than quantitative techniques (such as Western blot analysis) because an insufficient amount of protein would have been obtained from the microdissected veins for performance of such assays.

Analysis was performed for determination of the effects of exercise on collagen content of lung samples in this study because results of another study (38) indicate the amount of collagen in EIPH-affected lung tissue is higher than that in unaffected lung tissue. Results of the present study indicated that exercise did not have a significant effect on

collagen content of lung samples. However, collagen content was significantly different in tissue samples obtained from caudodorsal and cranioventral regions of lungs. Although areas of slides with pleural tissue were excluded from analysis, that finding was likely attributable to anatomic differences between caudodorsal and cranioventral regions of lungs. Also, expression of collagen type I mRNA was not affected by exercise of horses. Similar morphometric analysis for the proteins evaluated via immunohistochemical methods (TGF-β, VEGF and tenascin-C) was not performed because differences in expression of those proteins were not detected via routine microscopy.

Interactions among mediators of venous remodeling are complex and affected by the type and severity of a stimulus and the timing of tissue sample collection. For example, during development of TGF-β–mediated intimal hyperplasia in vein grafts in rabbits, activities of MMP-2 and MMP-9 concurrently decrease (85). Results of another study indicate there is a temporal pattern of MMP-2 and MMP-9 expression during venous remodeling, with an initial increase in expression followed by a decrease in expression to undetectable levels (195). The significant decrease in expression of MMP-2 and MMP-9 mRNA detected in the present study after exercise of horses may have been attributable to a period of blood vessel remodeling during which those substances had low expression.

A limitation of the present study was the fact that lung samples were evaluated for only one time after exercise of horses. Results of another study in which gene expression in autologous vein grafts was evaluated via high throughput microarray analysis (92) indicate expression of TIMP-1 and VEGF mRNA is increased only on day 1 after graft implantation, and not on days 7, 14, or 30 after graft implantation; results of that study also indicate collagen expression is decreased on days 1 and 7, and increased on days 14 and 30 after

graft implantation. Because data have not been published regarding gene expression in equine pulmonary veins, to the authors' knowledge, the timing of lung sample collection and the duration of exercise of horses in this study were selected on the basis of other information. Continuous hypertension causes substantial structural alterations in the tunica media and adventitia of pulmonary veins in sheep after only 4 days (86); therefore, we predicted that alterations in gene expression (which should precede structural alterations) in vein walls of horses in the present study would be detectable 2 weeks after the end of a 6-session intense exercise period. Because results of this study indicated mRNA expression of various MMPs and growth factors was significantly different after exercise versus gene expression before exercise, that duration and intensity of exercise for horses seemed to be adequate to cause changes in gene expression.

The high-intensity exercise protocol used in the present study was intended to simulate race training (after horses underwent 2 weeks of low-intensity conditioning exercise). The intensity of exercise was expected to be an adequate stimulus for evaluation of changes in gene expression in lungs of horses. Each horse exercised at a speed corresponding to a heart rate of 120% of the HR_{max} value. The variable HR_{max} is a reproducible measurement for exercising horses (45), and horses require maximum effort to maintain a position on a treadmill at a speed corresponding to 120% of HR_{max}. Furthermore, 33.3% of horses in this study had EIPH (as diagnosed via respiratory tract endoscopy); this finding suggested that the exercise was of adequate intensity.

There was a 12-month period between collection of pre- and postexercise lung samples in this study. This period allowed healing of surgical sites after the first procedure. Ageing of animals is associated with remodeling of blood vessel walls (66) (particularly

arterial walls (110)); however, such findings have only been detected for very young and very old animals (110) and humans (104, 155). Therefore, it was unlikely that ageing during the 12-month period affected blood vessel wall characteristics in horses in the present study.

The role of venous remodeling in the pathogenesis of EIPH is not known, to the authors' knowledge. However, the distribution of venous remodeling in lungs of horses with EIPH (lesions are colocalized with hemosiderin in caudodorsal regions of lungs of affected horses (38)) suggests that it is important in the pathogenesis of EIPH. Because high intravascular pressures induce remodeling in systemic (2, 28, 29, 64, 112, 180, 235) and pulmonary (25, 86) veins, we propose that intermittent periods of high pressures in the pulmonary circulation during exercise cause remodeling of pulmonary veins in caudodorsal regions of lungs of horses. Such venous remodeling may result in high pulmonary capillary pressures in affected regions of lungs and an increased risk of capillary rupture and hemorrhage and development of EIPH.

Results of the present study did not support the hypothesis that 2 weeks of intense exercise would cause alterations in gene and protein expression in pulmonary veins in a manner expected to favor venous remodeling. However, few data regarding timing of expression of genes during vascular remodeling in horses have been published. Further studies are warranted to determine the mechanisms and timing of venous remodeling in horses with EIPH.

APPENDIX

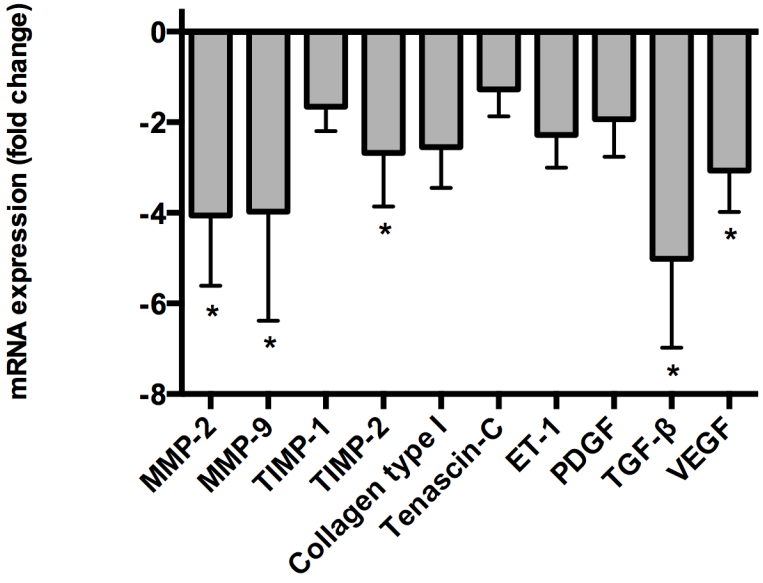

Figure 15: Mean ± SEM fold changes in mRNA expression of 10 genes in pulmonary vein samples of 4 horses after a 2-week period of intense exercise versus expression before exercise. *Expression is significantly ($P < 0.05$) different between pulmonary vein samples collected before and after exercise.

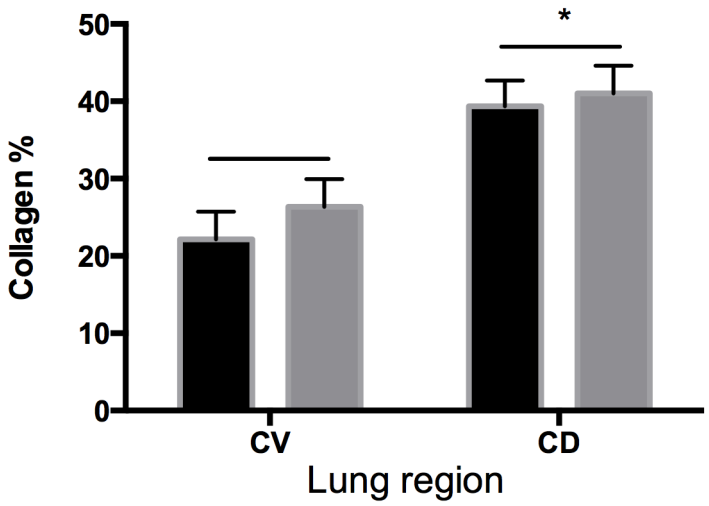

Figure 16: Least square mean ± SEM percentage of collagen in samples of cranioventral (CV) and caudodorsal (CD) regions of lung of 6 horses before (black bars) and after (grey bars) a 2-week period of intense exercise. Bars indicate no significant ($P < 0.05$) differences between lung samples obtained before and after exercise within a region. *Mean value for pre- and postexercise tissue samples obtained from caudodorsal regions of lungs are significantly ($P < 0.05$) higher than those obtained from cranioventral regions of lungs.

Gene	GenBank accession No.	Forward primer (5'–3')	Reverse primer (5'–3')	Probe
MMP-2	AJ010314	TCCGAGTCTGGAGTGATGTGA	GATCATGATGTCAGCCTCTCCAT	CCCACTACGGTTTTCT
MMP-9	NM-001111302	GCAAGGAGTACTCTGCCTGTA	CCAGAGGCGCCCATCA	CTGCGGCCCTCTCTG
TIMP-1	NM_001082515	GCCAGGGCTTCACCAAGA	CAGTGTCACTCTGCAGTTTGC	ATGCTCAGTGTTTCCC
TIMP-2	AJ010315	CTGACAAGGACATCGAGTTCATCTA	GCGAGACCCCGCACA	ACGGCTCCCTCCTCG
PDGF	XM001914920	GAGCCCAGAGCAGATGCAA	CTTCTTGCTCTGACCCACGAT	ACAGCAGCCCACTTGC
TGF-β	NM-001081849	GGAATGGCTGTCCTTTGATGTCA	CGAAGGCCCTCCATTGC	CTGCCGCACGACTCC
ET-1	AY730629	CGACATCATCTGGGTCAACACT	GGATCGCTTGGACCTGGAA	CCGAGCACATTGTTCC
Collagen type I	AF034691	CGGACAGCCTGGACTCC	CAGCAAATTTCTCATCATAGCCATAAGAC	CCTCCTGGACCTCCCG
VEGF	NM_001081821	GCAAATGTGAATGCAGACCAAAGAA	GCTTTCTCCGCTCTGAGCAA	CCACAGGGATTTTC
Tenascin-C	AY246747	GTGGAGTATTTCATCCGTGTGTTTG	GCCACCCTGGCACTGA	CCATCCCGGAGAACAA
Beta-actin	NM_001081838	GGGACCTGACGGACTACCT	CCGTGGTGGTGAAGCTGTAG	TCCGTGAGGATCTTCA
B2M	NM_001082502	CGCCTGAGATTGAAATTGATTTGCT	GACCAGTCCTTGCTGAAAGACA	ACCGGTCGACTTTCAT
EF-1	AY237113	CCACCAACTCGTCCAACTGATAAG	GACAGTACCGATACCACCAATTTTG	CCCTTGCGTCTGCCCC

Table 2 Primers and probes used for detection of various genes in pulmonary vein samples of horses via qRT-PCR assay. B2M = Beta-2-microglobulin. EF-1 = Elongation factor-1 alpha.

CHAPTER 5

Conclusions and directions for future studies

Recent descriptions of EIPH pathology have highlighted deficiencies in the capillary-stress failure theory of EIPH pathogenesis, which until recently provided the most plausible, albeit incomplete, explanation of disease mechanisms.

Pulmonary capillary stress failure secondary to exercise-associated pulmonary circulation pressure elevations explains neither the predilection for caudodorsal lung of EIPH pathology, the distribution of which matches exactly that of pulmonary blood flow during exercise, nor does it account for the extensive venous remodeling of intralobular pulmonary veins in caudodorsal lung. Assuming however that stress failure is a component of EIPH pathogenesis, and evidence exists to suggest that it is, the factors that determine capillary pressure, and therefore pulmonary capillary rupture, merit consideration.

Resistance to flow in a vessel is strongly influenced by vessel diameter, which in turn is a function of a combination of the passive, mechanical characteristics of the vessel wall (i.e. its ability to resist stretch) and the degree of contraction of circumferential vascular smooth muscle (i.e. vessel tone), which is determined by neural, humoral and local factors.

Capillary pressure is immediately influenced by the resistance to blood flow in the segments supplying and draining that capillary. Decreased arterial resistance to flow and increased venous resistance to flow are both conditions under which intervening capillaries will be exposed to higher pressures.

While there is evidence that regional heterogeneity in the reactivity of large pulmonary arteries in the horse lung exists, whether a similar pattern exists in the small

arteries and veins, that are almost immediately up- and down-stream from the pulmonary capillaries had not been investigated. I hypothesized therefore that regional differences do exist, in either the mechanical characteristics, and/or in the reactivity profile of these vessels, and that these differences would provide some evidence that capillary pressures in caudodorsal lung could exceed those in cranioventral lung during exercise. Furthermore, if those regional differences predict the transmission of higher pressures to pulmonary veins in the caudodorsal lung, then hemodynamic stimuli in that region, while transient, would be enough to initiate pressure-mediated venous remodeling. Remodeling would reduce venous compliance, and further exacerbate pulmonary capillary failure.

Accordingly, my overarching hypothesis for EIPH pathogenesis was as follows:

During intense exercise horses experience elevations in cardiac output that result in elevated pulmonary artery, left atrial and pulmonary capillary pressures. In caudodorsal regions of lung that already experience highest flow, regional differences in determinants of arterial and venous vessel diameter promote even higher pulmonary capillary pressures. Pulmonary capillary breaking strength is exceeded resulting in stress failure of some capillaries, and extravasation of red cells and airway hemorrhage. During both training and racing, repeated episodes of high pulmonary blood flow and pressures, particularly in the highest flow regions within caudodorsal lung, provide adequate hemodynamic stimuli to result in pulmonary venous remodeling. Remodeled pulmonary veins are less compliant than normal veins and failure of these vessels to distend normally further increases pulmonary capillary pressures. Those capillaries that are drained by remodeled veins are even more susceptible to rupture during exercise as venous wall compliance is reduced and in some cases, venous luminal area is diminished. With each exercise bout the injurious

cycle is repeated and compounded, ultimately resulting in clinically detectable hemorrhage, significant pulmonary pathology, and potentially impaired performance.

In the studies outlined in this dissertation I determined that regional differences in the mechanical characteristics of both arteries and veins in control, unraced horses exist. Specifically, caudodorsal arteries are stiffer than arteries from cranioventral lung, and the converse is true of veins. These differences do not necessarily predict that during exercise, caudodorsal pulmonary capillaries and as a result, veins will be exposed to higher pressures than those in cranioventral regions. In fact, less distensible caudodorsal arteries may even protect caudodorsal capillaries somewhat from transmission of the highest arterial pressures during exercise, and more compliant veins in this region also provide a degree of capillary protection. Of particular interest in this study however was the observation that pulmonary veins from caudodorsal lung of horses that had raced, but had neither a clinical history, nor pathologic evidence of severe EIPH, became significantly stiffer than those veins from control, unraced horses. Although structural components of the study vessels were not evaluated specifically, it is most likely that collagen deposition in vein walls such as has been reported in other studies contributed to the increase in elastic modulus of these vessels. These are the first data to demonstrate possible physiologic ramifications of venous remodeling in EIPH, namely reduced venous compliance.

While these data support the contention that venous remodeling is an early event in EIPH development, a two-week intermittent exercise stimuls was insufficient to induce changes in vein wall mRNA that would support initiation of remodeling at that stage.

The autonomic control of small pulmonary arteries and veins also demonstrates a regionally heterogeneous pattern. In the absence of any α-adrenoreceptor mediated vasoconstriction, β-adrenergic activity is expected to predominate in the high sympathetic outflow conditions experienced during exercise. A β-adrenergic agonist failed to cause relaxation of precontracted pulmonary veins, and relaxed pulmonary arteries from caudodorsal lung to a greater degree than those in cranioventral lung, a combination that in an *in* vivo setting could act to cause greater capillary pressures in caudodorsal lung. A muscarinic agonist caused pulmonary veins and caudodorsal arteries (mediated by both nitric oxide and prostanoid release) to relax, whereas caudodorsal arteries contracted. In the exercising horse and in the absence of parasympathetic input, the inverse of this pattern could serve to deliver the highest pulmonary capillary and venous pressures to caudodorsal regions.

In summary, regional difference in autonomic control of small pulmonary arteries and veins support the theory that the highest pulmonary capillary and venous pressures occur in the caudodorsal lung during exercise. Mechanical characteristics of the same vessels appear to reflect this regional pattern and in fact, vessel wall structure may "offset" these projected pressure differences somewhat. Probably as a result of vessel heterogeneity, venous remodeling in caudodorsal regions in response to hemodynamic stimuli associated with racing reduces venous compliance in a region-dependent manner, even before the development of severe pathology.

With regard to future directions for study, at this time I consider a more detailed characterization of the reactivity profiles of small pulmonary arteries and veins of the horse, in particular responses to naturally occurring agonists such as endothelin, and

serotonin a priority. This approach will add depth to current understanding of *in vivo* control of vessel tone, and provide direction for future investigations into pharmacotherapeutic interventions in EIPH. Although furosemide did not have a clinically relevant effect on pulmonary venous tone, testing this drug on larger pulmonary vessels, and preliminary investigations into other venous specific vasoactive agents, for example, C-type natriuretic peptide (209) is also warranted. Also, detailed, morphometric characterization of the changes in vessel wall structure that result in altered wall mechanical properties will provide another layer of understanding of the venous remodeling process. If this information were coupled with a detailed training/racing history it would permit elucidation of the exact nature of the stimulus required for mild/moderate/severe remodeling, and enable exploration of non-pharmacologic approaches to EIPH management along the lines of training modifications etc.

It is noteworthy that regional differences such as are described in these studies have not been reported in the pulmonary microvasculature of other species. Whether these differences occur in other species certainly merits further investigation at this time. I consider it unlikely that these observations are unique to the horse, and should they occur across other mammalian species, they have potential for application in a wide range of contexts, in particular in the study of pulmonary vascular pathology including pulmonary hypertension, pulmonary veno-occlusive disease and left heart failure, all of which are conditions associated with high morbidity rates (25, 122). Indeed, until recently, pulmonary veins were viewed by most as mere "conduit vessels" and as a result, are relatively understudied (50).

In conclusion, these data further cement the role of the pulmonary vein as a pivotal component of EIPH pathology and shed new light on features of the equine pulmonary circulation that place the exercising horse at risk of caudodorsal capillary rupture, and EIPH. Thus, these investigations provide evidence that support a new, comprehensive theory of EIPH pathogenesis, a framework for future study of EIPH, and novel contributions to pulmonary vascular biology literature.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. *General stud book, containing pedigrees of race horses, &c., from the earliest accounts*. Lond: C. & J. Weatherby, 1858, p. 18v.

2. **Abeles D, Kwei S, Stavrakis G, Zhang Y, Wang ET, and Garcia-Cardena G**. Gene expression changes evoked in a venous segment exposed to arterial flow. *J Vasc Surg* 44: 863-870, 2006.

3. **Aitsebaomo J, Portbury AL, Schisler JC, and Patterson C**. Brothers and sisters: molecular insights into arterial-venous heterogeneity. *Circ Res* 103: 929-939, 2008.

4. **Akbar SJ, Derksen FJ, Billah AM, and Werney U**. Exercise induced pulmonary haemorrhage in racing camels. *Vet Rec* 135: 624-625, 1994.

5. **al-Tinawi A, Clough AV, Harder DR, Linehan JH, Rickaby DA, and Dawson CA**. Distensibility of small veins of the dog lung. *Journal of applied physiology* 73: 2158-2165, 1992.

6. **Allen D, Jr., Clark ES, Moore JN, and Prasse KW**. Evaluation of equine digital Starling forces and hemodynamics during early laminitis. *Am J Vet Res* 51: 1930-1934, 1990.

7. **Angus JA, and Wright CE**. Techniques to study the pharmacodynamics of isolated large and small blood vessels. *Journal of pharmacological and toxicological methods* 44: 395-407, 2000.

8. **Araya O, Palma P, Salvi M, Bustamante H, and Vits L**. Endoscopic determination of exercise-induced pulmonary haemorrhage in Chilean Criollo horses. *Vet J* 169: 311-313, 2005.

9. **Banks J, Booth FV, MacKay EH, Rajagopalan B, and Lee GD**. The physcial properties of human pulmonary arteries and veins. *Clinical science and molecular medicine* 55: 477-484, 1978.

10. **Barman SA, Senteno E, Smith S, and Taylor AE**. Acetylcholine's effect on vascular resistance and compliance in the pulmonary circulation. *Journal of applied physiology* 67: 1495-1503, 1989.

11. **Barnes PJ, and Liu SF**. Regulation of pulmonary vascular tone. *Pharmacological reviews* 47: 87-131, 1995.

12. **Bashey RI, Cox R, McCann J, and Jimenez SA**. Changes in collagen biosynthesis, types, and mechanics of aorta in hypertensive rats. *J Lab Clin Med* 113: 604-611, 1989.

13. **Benamou AE, Marlin DJ, and Lekeux P**. Equine pulmonary and systemic haemodynamic responses to endothelin-1 and a selective ET(A) receptor antagonist. *Equine veterinary journal* 33: 337-344, 2001.

14. **Berceli SA, Jiang Z, Klingman NV, Pfahnl CL, Abouhamze ZS, Frase CD, Schultz GS, and Ozaki CK**. Differential expression and activity of matrix metalloproteinases during flow-modulated vein graft remodeling. *J Vasc Surg* 39: 1084-1090, 2004.

15. **Bernard SL, Glenny RW, Erickson HH, Fedde MR, Polissar N, Basaraba RJ, and Hlastala MP**. Minimal redistribution of pulmonary blood flow with exercise in racehorses. *J Appl Physiol* 81: 1062-1070, 1996.

16. **Birks EK, Mathieu-Costello O, Fu Z, Tyler WS, and West JB**. Comparative aspects of the strength of pulmonary capillaries in rabbit, dog, and horse. *Respir Physiol* 97: 235-246, 1994.

17. **Birks EK, Mathieu-Costello O, Fu Z, Tyler WS, and West JB**. Very high pressures are required to cause stress failure of pulmonary capillaries in thoroughbred racehorses. *J Appl Physiol* 82: 1584-1592, 1997.

18. **Birks EK, Shuler KM, Soma LR, Martin BB, Marconato L, Del Piero F, Teleis DC, Schar D, Hessinger AE, and Uboh CE**. EIPH: postrace endoscopic evaluation of Standardbreds and Thoroughbreds. *Equine Vet J Suppl* 375-378, 2002.

19. **Bronquard C, Maupoil V, Arbeille B, Fetissof F, Findlay I, Cosnay P, and Freslon JL**. Contractile and relaxant properties of rat-isolated pulmonary veins related to localization and histology. *Fundamental & clinical pharmacology* 21: 55-65, 2007.

20. **Burton AC**. Relation of structure to function of the tissues of the wall of blood vessels. *Physiol Rev* 34: 619-642, 1954.

21. **Buus CL, Pourageaud F, Fazzi GE, Janssen G, Mulvany MJ, and De Mey JG**. Smooth muscle cell changes during flow-related remodeling of rat mesenteric resistance arteries. *Circ Res* 89: 180-186, 2001.

22. **Caro CG, and Saffman PG**. Extensibility of Blood Vessels in Isolated Rabbit Lungs. *J Physiol* 178: 193-210, 1965.

23. **Castro MM, Rizzi E, Prado CM, Rossi MA, Tanus-Santos JE, and Gerlach RF**. Imbalance between matrix metalloproteinases and tissue inhibitor of metalloproteinases in hypertensive vascular remodeling. *Matrix Biol* 29: 194-201, 2010.

24. **Chay S, Woods WE, Rowse K, Nugent TE, Blake JW, and Tobin T**. The pharmacology of furosemide in the horse. V. Pharmacokinetics and blood levels of furosemide after intravenous administration. *Drug Metab Dispos* 11: 226-231, 1983.

25. **Chazova I, Robbins I, Loyd J, Newman J, Tapson V, Zhdaov V, and Meyrick B**. Venous and arterial changes in pulmonary veno-occlusive disease, mitral stenosis and fibrosing mediastinitis. *Eur Respir J* 15: 116-122, 2000.

26. **Chelladurai P, Seeger W, and Pullamsetti SS**. Matrix metalloproteinases and their inhibitors in pulmonary hypertension. *Eur Respir J* 40: 766-782, 2012.

27. **Chesler NC, Ku DN, and Galis ZS**. Transmural pressure induces matrix-degrading activity in porcine arteries ex vivo. *Am J Physiol* 277: H2002-2009, 1999.

28. **Choy JS, Dang Q, Molloi S, and Kassab GS**. Nonuniformity of axial and circumferential remodeling of large coronary veins in response to ligation. *Am J Physiol Heart Circ Physiol* 290: H1558-1565, 2006.

29. **Chung AW, Rauniyar P, Luo H, Hsiang YN, van Breemen C, and Okon EB**. Pressure distention compared with pharmacologic relaxation in vein grafting upregulates matrix metalloproteinase-2 and -9. *J Vasc Surg* 42: 747-756, 2005.

30. **Coflesky JT, Jones RC, Reid LM, and Evans JN**. Mechanical properties and structure of isolated pulmonary arteries remodeled by chronic hyperoxia. *Am Rev Respir Dis* 136: 388-394, 1987.

31. **Cohen MR, Hinsch E, Vergona R, Ryan J, Kolis SJ, and Schwartz MA**. A comparative diuretic and tissue distribution study of bumetanide and furosemide in the dog. *J Pharmacol Exp Ther* 197: 697-702, 1976.

32. **Cook WR**. Epistaxis in the racehorse. *Equine veterinary journal* 6: 45-58, 1974.

33. **Cox RH**. Comparison of mechanical and chemical properties of extra- and intralobar canine pulmonary arteries. *Am J Physiol* 242: H245-253, 1982.

34. **Dawson CA**. Role of pulmonary vasomotion in physiology of the lung. *Physiol Rev* 64: 544-616, 1984.

35. **Deacon K, and Knox AJ**. Endothelin-1 (ET-1) increases the expression of remodeling genes in vascular smooth muscle through linked calcium and cAMP pathways: role of a phospholipase A(2)(cPLA(2))/cyclooxygenase-2 (COX-2)/prostacyclin receptor-dependent autocrine loop. *J Biol Chem* 285: 25913-25927, 2010.

36. **Deem DA, and Fregin GF**. Atrial fibrillation in horses: a review of 106 clinical cases, with consideration of prevalence, clinical signs, and prognosis. *J Am Vet Med Assoc* 180: 261-265, 1982.

37. **deMello DE, and Reid LM**. Embryonic and early fetal development of human lung vasculature and its functional implications. *Pediatric and developmental pathology : the official journal of the Society for Pediatric Pathology and the Paediatric Pathology Society* 3: 439-449, 2000.

38. **Derksen FJ, Williams KJ, Pannirselvam RR, de Feijter-Rupp H, Steel CM, and Robinson NE**. Regional distribution of collagen and haemosiderin in the lungs of horses with exercise-induced pulmonary haemorrhage. *Equine veterinary journal* 41: 586-591, 2009.

39. **Derksen FJ, Williams KJ, Uhal BD, Slocombe RF, de Feijter-Rupp H, Eberhart S, Berney C, and Robinson NE**. Pulmonary response to airway instillation of autologous blood in horses. *Equine veterinary journal* 39: 334-339, 2007.

40. **Ding X, and Murray PA**. Cellular mechanisms of thromboxane A2-mediated contraction in pulmonary veins. *Am J Physiol Lung Cell Mol Physiol* 289: L825-833, 2005.

41. **Elliott FM, and Reid L**. Some New Facts About the Pulmonary Artery and Its Branching Pattern. *Clinical radiology* 16: 193-198, 1965.

42. **Epp TS, Szladovits B, Buchannan A, Gates L, McDonough P, Padilla DJ, Smart J, Erickson HH, and Poole DC**. Evidence supporting exercise-induced pulmonary haemorrhage in racing greyhounds. *Comparative Exercise Physiology* 5: 21-32, 2008.

43. **Erickson HH, Bernard SL, Glenny RW, Fedde MR, Polissar NL, Basaraba RJ, Walther SM, Gaughan EM, McMurphy R, and Hlastala MP**. Effect of furosemide on pulmonary blood flow distribution in resting and exercising horses. *Journal of applied physiology* 86: 2034-2043, 1999.

44. **Evans DL, and Rose RJ**. Cardiovascular and respiratory responses in Thoroughbred horses during treadmill exercise. *J Exp Biol* 134: 397-408, 1988.

45. **Evans DL, and Rose RJ**. Determination and repeatability of maximum oxygen uptake and other cardiorespiratory measurements in the exercising horse. *Equine veterinary journal* 20: 94-98, 1988.

46. **Ferrara N**. Vascular endothelial growth factor: basic science and clinical progress. *Endocr Rev* 25: 581-611, 2004.

47. **Fleige S, and Pfaffl MW**. RNA integrity and the effect on the real-time qRT-PCR performance. *Mol Aspects Med* 27: 126-139, 2006.

48. **Francis SE, Hunter S, Holt CM, Gadsdon PA, Rogers S, Duff GW, Newby AC, and Angelini GD**. Release of platelet-derived growth factor activity from pig venous arterial grafts. *J Thorac Cardiovasc Surg* 108: 540-548, 1994.

49. **Gaar KA, Jr., Taylor AE, Owens LJ, and Guyton AC**. Pulmonary capillary pressure and filtration coefficient in the isolated perfused lung. *Am J Physiol* 213: 910-914, 1967.

50. **Gao Y, and Raj JU**. Role of veins in regulation of pulmonary circulation. *Am J Physiol Lung Cell Mol Physiol* 288: L213-226, 2005.

51. **Gerbino AJ, Altemeier WA, Schimmel C, and Glenny RW**. Endotoxemia increases relative perfusion to dorsal-caudal lung regions. *Journal of applied physiology* 90: 1508-1515, 2001.

52. **Ghio AJ, Ghio C, and Bassett M**. Exercise-induced pulmonary hemorrhage after running a marathon. *Lung* 184: 331-333, 2006.

53. **Ghofrani HA, Seeger W, and Grimminger F**. Imatinib for the treatment of pulmonary arterial hypertension. *N Engl J Med* 353: 1412-1413, 2005.

54. **Gleed FD, Ducharme NG, Hackett RP, Hakim TS, Erb HN, Mitchell LM, and Soderholm LV**. Effects of frusemide on pulmonary capillary pressure in horses exercising on a treadmill. *Equine Vet J Suppl* 102-106, 1999.

55. **Glenny RW**. Blood flow distribution in the lung. *Chest* 114: 8S-16S, 1998.

56. **Glenny RW, Bernard S, Robertson HT, and Hlastala MP**. Gravity is an important but secondary determinant of regional pulmonary blood flow in upright primates. *Journal of applied physiology* 86: 623-632, 1999.

57. **Glenny RW, Lamm WJ, Albert RK, and Robertson HT**. Gravity is a minor determinant of pulmonary blood flow distribution. *Journal of applied physiology* 71: 620-629, 1991.

58. **Goumans MJ, Liu Z, and ten Dijke P**. TGF-beta signaling in vascular biology and dysfunction. *Cell research* 19: 116-127, 2009.

59. **Greenberg S, McGowan C, Xie J, and Summer WR**. Selective pulmonary and venous smooth muscle relaxation by furosemide: a comparison with morphine. *J Pharmacol Exp Ther* 270: 1077-1085, 1994.

60. **Gross DK, Morley PS, Hinchcliff KW, and Wittum TE**. Effect of furosemide on performance of Thoroughbreds racing in the United States and Canada. *J Am Vet Med Assoc* 215: 670-675, 1999.

61. **Gusic RJ, Myung R, Petko M, Gaynor JW, and Gooch KJ**. Shear stress and pressure modulate saphenous vein remodeling ex vivo. *Journal of biomechanics* 38: 1760-1769, 2005.

62. **Halpern W, Mulvany MJ, and Warshaw DM**. Mechanical properties of smooth muscle cells in the walls of arterial resistance vessels. *J Physiol* 275: 85-101, 1978.

63. **Hanna CJ, and Eyre P**. Pharmacological studies on the pulmonary vein of the horse. I. Effects of selected spasmogens. *Canadian journal of physiology and pharmacology* 56: 812-817, 1978.

64. **Hayashi K, Mori K, and Miyazaki H**. Biomechanical response of femoral vein to chronic elevation of blood pressure in rabbits. *Am J Physiol Heart Circ Physiol* 284: H511-518, 2003.

65. **Heldin CH, and Westermark B**. Mechanism of action and in vivo role of platelet-derived growth factor. *Physiol Rev* 79: 1283-1316, 1999.

66. **Hemmeryckx B, Emmerechts J, Bovill EG, Hoylaerts MF, and Lijnen HR**. Effect of ageing on the murine venous circulation. *Histochem Cell Biol* 137: 537-546, 2012.

67. **Herity NA, Ward MR, Lo S, and Yeung AC**. Review: Clinical aspects of vascular remodeling. *J Cardiovasc Electrophysiol* 10: 1016-1024, 1999.

68. **Hillidge CJ, and Whitlock TW**. Sex variation in the prevalence of exercise-induced pulmonary haemorrhage in racing quarter horses. *Research in veterinary science* 40: 406-407, 1986.

69. **Hinchcliff KW, Jackson MA, Brown JA, Dredge AF, O'Callaghan PA, McCaffrey JP, Morley PS, Slocombe RE, and Clarke AF**. Tracheobronchoscopic assessment of exercise-induced pulmonary hemorrhage in horses. *Am J Vet Res* 66: 596-598, 2005.

70. **Hinchcliff KW, Jackson MA, Morley PS, Brown JA, Dredge AE, O'Callaghan PA, McCaffrey JP, Slocombe RE, and Clarke AE**. Association between exercise-induced pulmonary hemorrhage and performance in Thoroughbred racehorses. *J Am Vet Med Assoc* 227: 768-774, 2005.

71. **Hinchcliff KW, and McKeever KH**. Fluid administration attenuates the haemodynamic effect of frusemide in running horses. *Equine veterinary journal* 30: 246-250, 1998.

72. **Hinchcliff KW, McKeever KH, and Muir WW, 3rd**. Furosemide-induced changes in plasma and blood volume of horses. *J Vet Pharmacol Ther* 14: 411-417, 1991.

73. **Hinchcliff KW, Morley PS, and Guthrie A**. Use of furosemide for exercise-induced pulmonary hemorrhage in racehorses. *J Am Vet Med Assoc* 239: 1407, 2011.

74. **Hinchcliff KW, Morley PS, and Guthrie AJ**. Efficacy of furosemide for prevention of exercise-induced pulmonary hemorrhage in Thoroughbred racehorses. *J Am Vet Med Assoc* 235: 76-82, 2009.

75. **Hinchcliff KW, Morley PS, Jackson MA, Brown JA, Dredge AF, O'Callaghan PA, McCaffrey JP, Slocombe RF, and Clarke AF**. Risk factors for exercise-induced pulmonary haemorrhage in Thoroughbred racehorses. *Equine Vet J Suppl* 228-234, 2010.

76. **Hlastala MP, Bernard SL, Erickson HH, Fedde MR, Gaughan EM, McMurphy R, Emery MJ, Polissar N, and Glenny RW**. Pulmonary blood flow distribution in standing horses is not dominated by gravity. *J Appl Physiol* 81: 1051-1061, 1996.

77. **Hlastala MP, and Glenny RW**. Vascular structure determines pulmonary blood flow distribution. *News in physiological sciences : an international journal of physiology produced jointly by the International Union of Physiological Sciences and the American Physiological Society* 14: 182-186, 1999.

78. **Holcomb BW, Jr., Loyd JE, Ely EW, Johnson J, and Robbins IM**. Pulmonary veno-occlusive disease: a case series and new observations. *Chest* 118: 1671-1679, 2000.

79. **Hopkins SR, Schoene RB, Henderson WR, Spragg RG, Martin TR, and West JB**. Intense exercise impairs the integrity of the pulmonary blood-gas barrier in elite athletes. *Am J Respir Crit Care Med* 155: 1090-1094, 1997.

80. **Huang W, Yen RT, McLaurine M, and Bledsoe G**. Morphometry of the human pulmonary vasculature. *Journal of applied physiology* 81: 2123-2133, 1996.

81. **Huertas A, Girerd B, Dorfmuller P, O'Callaghan D, Humbert M, and Montani D**. Pulmonary veno-occlusive disease: advances in clinical management and treatments. *Expert review of respiratory medicine* 5: 217-229; quiz 230-211, 2011.

82. **Hunt JM, Bethea B, Liu X, Gandjeva A, Mammen PP, Stacher E, Gandjeva MR, Parish E, Perez M, Smith L, Graham BB, Kuebler WM, and Tuder RM**. Pulmonary veins in the normal lung and pulmonary hypertension due to left heart disease. *Am J Physiol Lung Cell Mol Physiol* 305: L725-736, 2013.

83. **Hyman AL, Lippton HL, and Kadowitz PJ**. Autonomic regulation of the pulmonary circulation. *J Cardiovasc Pharmacol* 7 Suppl 3: S80-95, 1985.

84. **Ibe BO, Hillyard RM, and Raj JU**. Heterogeneity in prostacyclin and thromboxane synthesis in ovine pulmonary vascular tree: effect of age and oxygen tension. *Exp Lung Res* 22: 351-374, 1996.

85. **Jiang Z, Tao M, Omalley KA, Wang D, Ozaki CK, and Berceli SA**. Established neointimal hyperplasia in vein grafts expands via TGF-beta-mediated progressive fibrosis. *Am J Physiol Heart Circ Physiol* 297: H1200-1207, 2009.

86. **Johnson JE, Perkett EA, and Meyrick B**. Pulmonary veins and bronchial vessels undergo remodeling in sustained pulmonary hypertension induced by continuous air embolization into sheep. *Exp Lung Res* 23: 459-473, 1997.

87. **Johnson LR, Parker JL, and Laughlin MH**. Chronic exercise training improves ACh-induced vasorelaxation in pulmonary arteries of pigs. *Journal of applied physiology* 88: 443-451, 2000.

88. **Jones JH, Smith BL, Birks EK, Pascoe JR, and Hughes TR**. LEFT ATRIAL AND PULMONARY ARTERIAL PRESSURES IN EXERCISING HORSES. *Faseb Journal* 6: A2020-A2020, 1992.

89. **Jones PL, Crack J, and Rabinovitch M**. Regulation of tenascin-C, a vascular smooth muscle cell survival factor that interacts with the alpha v beta 3 integrin to promote epidermal growth factor receptor phosphorylation and growth. *J Cell Biol* 139: 279-293, 1997.

90. **Junbao D, Hui Y, Bing W, Jian L, Jianguang Q, and Chaoshu T**. Effect of L-arginine on collagen of high flow-induced pulmonary arterial remodeling. *Circulation journal : official journal of the Japanese Circulation Society* 69: 603-608, 2005.

91. **Kadowitz PJ, and Hyman AL**. Effect of sympathetic nerve stimulation on pulmonary vascular resistance in the dog. *Circ Res* 32: 221-227, 1973.

92. **Kalish JA, Willis DJ, Li C, Link JJ, Deutsch ER, Contreras MA, Quist WC, and Logerfo FW**. Temporal genomics of vein bypass grafting through oligonucleotide microarray analysis. *J Vasc Surg* 39: 645-654, 2004.

93. **Kane DW, Tesauro T, and Newman JH**. Adrenergic modulation of the pulmonary circulation during strenuous exercise in sheep. *Am Rev Respir Dis* 147: 1233-1238, 1993.

94. **Karau KL, Johnson RH, Molthen RC, Dhyani AH, Haworth ST, Hanger CC, Roerig DL, and Dawson CA**. Microfocal X-ray CT imaging and pulmonary arterial distensibility in excised rat lungs. *Am J Physiol Heart Circ Physiol* 281: H1447-1457, 2001.

95. **Kay JM**. Comparative morphologic features of the pulmonary vasculature in mammals. *Am Rev Respir Dis* 128: S53-57, 1983.

96. **Kedzierski RM, and Yanagisawa M**. Endothelin system: the double-edged sword in health and disease. *Annual review of pharmacology and toxicology* 41: 851-876, 2001.

97. **Keen JA, Hillier C, McGorum BC, Bunton D, and Nally JE**. Evaluation of equine laminar vein function: harvesting, dissection and the use of functional methods to distinguish between veins and arteries. *Journal of pharmacological and toxicological methods* 57: 92-99, 2008.

98. **Kelly DA, Hislop AA, Hall SM, and Haworth SG**. Relationship between structural remodeling and reactivity in pulmonary resistance arteries from hypertensive piglets. *Pediatric research* 58: 525-530, 2005.

99. **Kemp BK, Smolich JJ, and Cocks TM**. Evidence for specific regional patterns of responses to different vasoconstrictors and vasodilators in sheep isolated pulmonary arteries and veins. *Br J Pharmacol* 121: 441-450, 1997.

100. **Kindig CA, McDonough P, Finley MR, Behnke BJ, Richardson TE, Marlin DJ, Erickson HH, and Poole DC**. NO inhalation reduces pulmonary arterial pressure but not hemorrhage in maximally exercising horses. *Journal of applied physiology* 91: 2674-2678, 2001.

101. **Kobs RW, Muvarak NE, Eickhoff JC, and Chesler NC**. Linked mechanical and biological aspects of remodeling in mouse pulmonary arteries with hypoxia-induced hypertension. *Am J Physiol Heart Circ Physiol* 288: H1209-1217, 2005.

102. **Krenz GS, and Dawson CA**. Flow and pressure distributions in vascular networks consisting of distensible vessels. *Am J Physiol Heart Circ Physiol* 284: H2192-2203, 2003.

103. **Kuhl PG, Bolds JM, Loyd JE, Snapper JR, and FitzGerald GA**. Thromboxane receptor-mediated bronchial and hemodynamic responses in ovine endotoxemia. *Am J Physiol* 254: R310-319, 1988.

104. **Lakatta EG**. Age-associated cardiovascular changes in health: impact on cardiovascular disease in older persons. *Heart failure reviews* 7: 29-49, 2002.

105. **Lambers C, Roth M, Zhong J, Campregher C, Binder P, Burian B, Petkov V, and Block LH**. The interaction of endothelin-1 and TGF-beta1 mediates vascular cell remodeling. *PLoS One* 8: e73399, 2013.

106. **Langsetmo I, Meyer MR, and Erickson HH**. Relationship of pulmonary arterial pressure to pulmonary haemorrhage in exercising horses. *Equine veterinary journal* 32: 379-384, 2000.

107. **Lapointe JM, Vrins A, and McCarvill E**. A survey of exercise-induced pulmonary haemorrhage in Quebec standardbred racehorses. *Equine veterinary journal* 26: 482-485, 1994.

108. **Leach RM, Twort CH, Cameron IR, and Ward JP**. A comparison of the pharmacological and mechanical properties in vitro of large and small pulmonary arteries of the rat. *Clin Sci (Lond)* 82: 55-62, 1992.

109. **Lehoux S, Lemarie CA, Esposito B, Lijnen HR, and Tedgui A**. Pressure-induced matrix metalloproteinase-9 contributes to early hypertensive remodeling. *Circulation* 109: 1041-1047, 2004.

110. **Li Z, Froehlich J, Galis ZS, and Lakatta EG**. Increased expression of matrix metalloproteinase-2 in the thickened intima of aged rats. *Hypertension* 33: 116-123, 1999.

111. **Lichtinghagen R, Musholt PB, Lein M, Romer A, Rudolph B, Kristiansen G, Hauptmann S, Schnorr D, Loening SA, and Jung K**. Different mRNA and protein expression of matrix metalloproteinases 2 and 9 and tissue inhibitor of metalloproteinases 1 in benign and malignant prostate tissue. *Eur Urol* 42: 398-406, 2002.

112. **Liu SQ, and Fung YC**. Changes in the organization of the smooth muscle cells in rat vein grafts. *Ann Biomed Eng* 26: 86-95, 1998.

113. **Livak KJ, and Schmittgen TD**. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25: 402-408, 2001.

114. **Lovering AT, Romer LM, Haverkamp HC, Pegelow DF, Hokanson JS, and Eldridge MW**. Intrapulmonary shunting and pulmonary gas exchange during normoxic and hypoxic exercise in healthy humans. *Journal of applied physiology* 104: 1418-1425, 2008.

115. **Lugo J, Stick JA, Peroni J, Harkema JR, Derksen FJ, and Robinson NE**. Safety and efficacy of a technique for thoracoscopically guided pulmonary wedge resection in horses. *Am J Vet Res* 63: 1232-1240, 2002.

116. **Lundergan CF, Fitzpatrick TM, Rose JC, Ramwell PW, and Kot PA**. Effect of cyclooxygenase inhibition on the pulmonary vasodilator response to furosemide. *J Pharmacol Exp Ther* 246: 102-106, 1988.

117. **MacEachern KE, Smith GL, and Nolan AM**. Characteristics of the in vitro hypoxic pulmonary vasoconstrictor response in isolated equine and bovine pulmonary arterial rings. *Veterinary anaesthesia and analgesia* 31: 239-249, 2004.

118. **Machado RD, Aldred MA, James V, Harrison RE, Patel B, Schwalbe EC, Gruenig E, Janssen B, Koehler R, Seeger W, Eickelberg O, Olschewski H, Elliott CG, Glissmeyer E, Carlquist J, Kim M, Torbicki A, Fijalkowska A, Szewczyk G, Parma J, Abramowicz MJ, Galie N, Morisaki H, Kyotani S, Nakanishi N, Morisaki T, Humbert M, Simonneau G, Sitbon O, Soubrier F, Coulet F, Morrell NW, and Trembath RC**. Mutations of the TGF-beta type II receptor BMPR2 in pulmonary arterial hypertension. *Human mutation* 27: 121-132, 2006.

119. **Mackay EH, Banks J, Sykes B, and Lee G**. Structural basis for the changing physical properties of human pulmonary vessels with age. *Thorax* 33: 335-344, 1978.

120. **Malmstrom J, Lindberg H, Lindberg C, Bratt C, Wieslander E, Delander EL, Sarnstrand B, Burns JS, Mose-Larsen P, Fey S, and Marko-Varga G**. Transforming growth factor-beta 1 specifically induce proteins involved in the myofibroblast contractile apparatus. *Molecular & cellular proteomics : MCP* 3: 466-477, 2004.

121. **Maloney JE, Rooholamini SA, and Wexler L**. Pressure-diameter relations of small blood vessels in isolated dog lung. *Microvascular research* 2: 1-12, 1970.

122. **Mandel J, Mark EJ, and Hales CA**. Pulmonary veno-occlusive disease. *Am J Respir Crit Care Med* 162: 1964-1973, 2000.

123. **Manohar M**. Furosemide attenuates the exercise-induced increase in pulmonary artery wedge pressure in horses. *Am J Vet Res* 54: 952-958, 1993.

124. **Manohar M**. Pulmonary artery wedge pressure increases with high-intensity exercise in horses. *Am J Vet Res* 54: 142-146, 1993.

125. **Manohar M, and Goetz TE**. Intrapulmonary arteriovenous shunts of >15 microm in diameter probably do not contribute to arterial hypoxemia in maximally exercising Thoroughbred horses. *Journal of applied physiology* 99: 224-229, 2005.

126. **Manohar M, and Goetz TE**. L-NAME does not affect exercise-induced pulmonary hypertension in thoroughbred horses. *Journal of applied physiology* 84: 1902-1908, 1998.

127. **Manohar M, and Goetz TE**. Pulmonary vascular pressures of exercising thoroughbred horses with and without endoscopic evidence of EIPH. *J Appl Physiol* 81: 1589-1593, 1996.

128. **Manohar M, and Goetz TE**. Pulmonary vascular resistance of horses decreases with moderate exercise and remains unchanged as workload is increased to maximal exercise. *Equine Vet J Suppl* 117-121, 1999.

129. **Manohar M, Goetz TE, Sullivan E, and Griffin R**. Pulmonary vascular pressures of strenuously exercising Thoroughbreds after administration of varying doses of frusemide. *Equine veterinary journal* 29: 298-304, 1997.

130. **Markham G**. *Markham's masterpiece containing all knowledge belonging to the smith, farrier, or horse-leech, touching the curing of all diseases in horses ... being divided into two books, the first containing all cures physical, the second all belonging to chirurgery*. London: Printed at London by Edward Okes, and are to be sold by Thomas Passenger ... 1668, p. 13 , 591, 523 p.

131. **McKane SA, Canfield PJ, and Rose RJ**. Equine bronchoalveolar lavage cytology: survey of thoroughbred racehorses in training. *Australian veterinary journal* 70: 401-404, 1993.

132. **McLaughlin RF, Tyler WS, and Canada RO**. A study of the subgross pulmonary anatomy in various mammals. *American Journal of Anatomy* 108: 149-165, 1961.

133. **Merkus D, de Beer VJ, Houweling B, and Duncker DJ**. Control of pulmonary vascular tone during exercise in health and pulmonary hypertension. *Pharmacology & therapeutics* 119: 242-263, 2008.

134. **Michel RP**. Arteries and veins of the normal dog lung: qualitative and quantitative structural differences. *The American journal of anatomy* 164: 227-241, 1982.

135. **Mills PC, Marlin DJ, Demoncheaux E, Scott C, Casas I, Smith NC, and Higenbottam T**. Nitric oxide and exercise in the horse. *J Physiol* 495 (Pt 3): 863-874, 1996.

136. **Miyauchi T, and Masaki T**. Pathophysiology of endothelin in the cardiovascular system. *Annu Rev Physiol* 61: 391-415, 1999.

137. **Moses VS, and Bertone AL**. Nonsteroidal anti-inflammatory drugs. *The Veterinary clinics of North America Equine practice* 18: 21-37, v, 2002.

138. **Mulvany MJ, and Halpern W**. Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ Res* 41: 19-26, 1977.

139. **Mulvany MJ, and Halpern W**. Mechanical properties of vascular smooth muscle cells in situ. *Nature* 260: 617-619, 1976.

140. **Murray PA, Lodato RF, and Michael JR**. Neural antagonists modulate pulmonary vascular pressure-flow plots in conscious dogs. *Journal of applied physiology* 60: 1900-1907, 1986.

141. **Naeije R, and Chesler N**. Pulmonary circulation at exercise. *Comprehensive Physiology* 2: 711-741, 2012.

142. **Nagase H, Visse R, and Murphy G**. Structure and function of matrix metalloproteinases and TIMPs. *Cardiovasc Res* 69: 562-573, 2006.

143. **Nandiwada PA, Hyman AL, and Kadowitz PJ**. Pulmonary vasodilator responses to vagal stimulation and acetylcholine in the cat. *Circ Res* 53: 86-95, 1983.

144. **Newman JH, Butka BJ, and Brigham KL**. Thromboxane A2 and prostacyclin do not modulate pulmonary hemodynamics during exercise in sheep. *Journal of applied physiology* 61: 1706-1711, 1986.

145. **Newton JR, Rogers K, Marlin DJ, Wood JL, and Williams RB**. Risk factors for epistaxis on British racecourses: evidence for locomotory impact-induced trauma contributing to the aetiology of exercise-induced pulmonary haemorrhage. *Equine veterinary journal* 37: 402-411, 2005.

146. **Nikol S, Huehns TY, Weir L, Wight TN, and Hofling B**. Restenosis in human vein bypass grafts. *Atherosclerosis* 139: 31-39, 1998.

147. **Norel X**. Prostanoid receptors in the human vascular wall. *TheScientificWorldJournal* 7: 1359-1374, 2007.

148. **Norel X, Walch L, Costantino M, Labat C, Gorenne I, Dulmet E, Rossi F, and Brink C**. M1 and M3 muscarinic receptors in human pulmonary arteries. *Br J Pharmacol* 119: 149-157, 1996.

149. **Norel X, Walch L, Gascard JP, deMontpreville V, and Brink C**. Prostacyclin release and receptor activation: differential control of human pulmonary venous and arterial tone. *Br J Pharmacol* 142: 788-796, 2004.

150. **O'Callaghan MW, Pascoe JR, Tyler WS, and Mason DK**. Exercise-induced pulmonary haemorrhage in the horse: results of a detailed clinical, post mortem and imaging study. II. Gross lung pathology. *Equine veterinary journal* 19: 389-393, 1987.

151. **O'Callaghan MW, Pascoe JR, Tyler WS, and Mason DK**. Exercise-induced pulmonary haemorrhage in the horse: results of a detailed clinical, post mortem and imaging study. III. Subgross findings in lungs subjected to latex perfusions of the bronchial and pulmonary arteries. *Equine veterinary journal* 19: 394-404, 1987.

152. **O'Callaghan MW, Pascoe JR, Tyler WS, and Mason DK**. Exercise-induced pulmonary haemorrhage in the horse: results of a detailed clinical, post mortem and imaging study. V. Microscopic observations. *Equine veterinary journal* 19: 411-418, 1987.

153. **O'Callaghan MW, Pascoe JR, Tyler WS, and Mason DK**. Exercise-induced pulmonary haemorrhage in the horse: results of a detailed clinical, post mortem and imaging study. VIII. Conclusions and implications. *Equine veterinary journal* 19: 428-434, 1987.

154. **O'Donnell SR, and Wanstall JC**. Demonstration of both beta 1- and beta 2-adrenoceptors mediating relaxation of isolated ring preparations of rat pulmonary artery. *Br J Pharmacol* 74: 547-552, 1981.

155. **O'Leary DH, Polak JF, Kronmal RA, Manolio TA, Burke GL, and Wolfson SK, Jr.** Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults. Cardiovascular Health Study Collaborative Research Group. *N Engl J Med* 340: 14-22, 1999.

156. **Ohtani K, Egashira K, Hiasa K, Zhao Q, Kitamoto S, Ishibashi M, Usui M, Inoue S, Yonemitsu Y, Sueishi K, Sata M, Shibuya M, and Sunagawa K**. Blockade of vascular endothelial growth factor suppresses experimental restenosis after intraluminal injury by inhibiting recruitment of monocyte lineage cells. *Circulation* 110: 2444-2452, 2004.

157. **Oikawa M**. Exercise-induced haemorrhagic lesions in the dorsocaudal extremities of the caudal lobes of the lungs of young thoroughbred horses. *J Comp Pathol* 121: 339-347, 1999.

158. **Olsen SC, Coyne CP, Lowe BS, Pelletier N, Raub EM, and Erickson HH**. Influence of furosemide on hemodynamic responses during exercise in horses. *Am J Vet Res* 53: 742-747, 1992.

159. **Papaioannou TG, and Stefanadis C**. Vascular wall shear stress: basic principles and methods. *Hellenic journal of cardiology : HJC = Hellenike kardiologike epitheorese* 46: 9-15, 2005.

160. **Pappenheimer JR, and Soto-Rivera A**. Effective osmotic pressure of the plasma proteins and other quantities associated with the capillary circulation in the hindlimbs of cats and dogs. *Am J Physiol* 152: 471-491, 1948.

161. **Pardali E, Goumans MJ, and ten Dijke P**. Signaling by members of the TGF-beta family in vascular morphogenesis and disease. *Trends Cell Biol* 20: 556-567, 2010.

162. **Pascoe JR, Ferraro GL, Cannon JH, Arthur RM, and Wheat JD**. Exercise-induced pulmonary hemorrhage in racing thoroughbreds: a preliminary study. *Am J Vet Res* 42: 703-707, 1981.

163. **Passerini AG, Milsted A, and Rittgers SE**. Shear stress magnitude and directionality modulate growth factor gene expression in preconditioned vascular endothelial cells. *J Vasc Surg* 37: 182-190, 2003.

164. **Pelletier N, Robinson NE, Kaiser L, and Derksen FJ**. Regional differences in endothelial function in horse lungs: possible role in blood flow distribution? *J Appl Physiol* 85: 537-542, 1998.

165. **Pepke-Zaba J, Higenbottam TW, Dinh-Xuan AT, Ridden C, and Kealey T**. Alpha-adrenoceptor stimulation of porcine pulmonary arteries. *Eur J Pharmacol* 235: 169-175, 1993.

166. **Perros F, Montani D, Dorfmuller P, Durand-Gasselin I, Tcherakian C, Le Pavec J, Mazmanian M, Fadel E, Mussot S, Mercier O, Herve P, Emilie D, Eddahibi S, Simonneau G, Souza R, and Humbert M**. Platelet-derived growth factor expression and function in idiopathic pulmonary arterial hypertension. *Am J Respir Crit Care Med* 178: 81-88, 2008.

167. **Pertovaara L, Kaipainen A, Mustonen T, Orpana A, Ferrara N, Saksela O, and Alitalo K**. Vascular endothelial growth factor is induced in response to transforming growth factor-beta in fibroblastic and epithelial cells. *J Biol Chem* 269: 6271-6274, 1994.

168. **Pfister SL, Deinhart DD, and Campbell WB**. Methacholine-induced contraction of rabbit pulmonary artery: role of platelet-endothelial transcellular thromboxane synthesis. *Hypertension* 31: 206-212, 1998.

169. **Pick R, Jalil JE, Janicki JS, and Weber KT**. The fibrillar nature and structure of isoproterenol-induced myocardial fibrosis in the rat. *Am J Pathol* 134: 365-371, 1989.

170. **Pickkers P, Dormans TP, Russel FG, Hughes AD, Thien T, Schaper N, and Smits P**. Direct vascular effects of furosemide in humans. *Circulation* 96: 1847-1852, 1997.

171. **Raffetto JD, and Khalil RA**. Matrix metalloproteinases and their inhibitors in vascular remodeling and vascular disease. *Biochem Pharmacol* 75: 346-359, 2008.

172. **Raphel CF, and Soma LR**. Exercise-induced pulmonary hemorrhage in Thoroughbreds after racing and breezing. *Am J Vet Res* 43: 1123-1127, 1982.

173. **Reeves JT, Groves BM, Cymerman A, Sutton JR, Wagner PD, Turkevich D, and Houston CS**. Operation Everest II: cardiac filling pressures during cycle exercise at sea level. *Respir Physiol* 80: 147-154, 1990.

174. **Reeves JT, Linehan JH, and Stenmark KR**. Distensibility of the normal human lung circulation during exercise. *Am J Physiol Lung Cell Mol Physiol* 288: L419-425, 2005.

175. **Rhodin JA**. Microscopic anatomy of the pulmonary vascular bed in the cat lung. *Microvascular research* 15: 169-193, 1978.

176. **Rimeika D, Wiklund NP, Lindahl SG, and Wiklund CU**. Regional differences in nitric oxide-mediated vasorelaxation in porcine pulmonary arteries. *Acta Anaesthesiol Scand* 50: 947-953, 2006.

177. **Roach MR, and Burton AC**. The reason for the shape of the distensibility curves of arteries. *Canadian journal of biochemistry and physiology* 35: 681-690, 1957.

178. **Robinson NE, and Sorenson PR**. Pathophysiology of airway obstruction in horses: a review. *J Am Vet Med Assoc* 172: 299-303, 1978.

179. **Roy CS**. The Elastic Properties of the Arterial Wall. *J Physiol* 3: 125-159, 1881.

180. **Saucy F, Probst H, Alonso F, Berard X, Deglise S, Dunoyer-Geindre S, Mazzolai L, Kruithof E, Haefliger JA, and Corpataux JM**. Ex vivo pulsatile perfusion of human saphenous veins induces intimal hyperplasia and increased levels of the plasminogen activator inhibitor 1. *Eur Surg Res* 45: 50-59, 2010.

181. **Schermuly RT, Dony E, Ghofrani HA, Pullamsetti S, Savai R, Roth M, Sydykov A, Lai YJ, Weissmann N, Seeger W, and Grimminger F**. Reversal of experimental pulmonary hypertension by PDGF inhibition. *J Clin Invest* 115: 2811-2821, 2005.

182. **Schindler C, Dobrev D, Grossmann M, Francke K, Pittrow D, and Kirch W**. Mechanisms of beta-adrenergic receptor-mediated venodilation in humans. *Clinical pharmacology and therapeutics* 75: 49-59, 2004.

183. **Schroeder A, Mueller O, Stocker S, Salowsky R, Leiber M, Gassmann M, Lightfoot S, Menzel W, Granzow M, and Ragg T**. The RIN: an RNA integrity number for assigning integrity values to RNA measurements. *Bmc Mol Biol* 7: 3, 2006.

184. **Schroter RC, Marlin DJ, and Denny E**. Exercise-induced pulmonary haemorrhage (EIPH) in horses results from locomotory impact induced trauma--a novel, unifying concept. *Equine veterinary journal* 30: 186-192, 1998.

185. **Shaw AM, Bunton DC, Fisher A, McGrath JC, Montgomery I, Daly C, and MacDonald A**. V-shaped cushion at the origin of bovine pulmonary supernumerary arteries: structure and putative function. *Journal of applied physiology* 87: 2348-2356, 1999.

186. **Shebl FM, Pinto LA, Garcia-Pineres A, Lempicki R, Williams M, Harro C, and Hildesheim A**. Comparison of mRNA and protein measures of cytokines following vaccination with human papillomavirus-16 L1 virus-like particles. *Cancer Epidemiol Biomarkers Prev* 19: 978-981, 2010.

187. **Shi W, Eidelman DH, and Michel RP**. Differential relaxant responses of pulmonary arteries and veins in lung explants of guinea pigs. *Journal of applied physiology* 83: 1476-1481, 1997.

188. **Shi Y, Patel S, Niculescu R, Chung W, Desrochers P, and Zalewski A**. Role of matrix metalloproteinases and their tissue inhibitors in the regulation of coronary cell migration. *Arterioscler Thromb Vasc Biol* 19: 1150-1155, 1999.

189. **Shibamoto T, Wang HG, Yamaguchi Y, Hayashi T, Saeki Y, Tanaka S, and Koyama S**. Effects of thromboxane A2 analogue on vascular resistance distribution and permeability in isolated blood-perfused dog lungs. *Lung* 173: 209-221, 1995.

190. **Sinha AK, Gleed RD, Hakim TS, Dobson A, and Shannon KJ**. Pulmonary capillary pressure during exercise in horses. *Journal of applied physiology* 80: 1792-1798, 1996.

191. **Snow DH, Harris RC, MacDonald IA, Forster CD, and Marlin DJ**. Effects of high-intensity exercise on plasma catecholamines in the thoroughbred horse. *Equine veterinary journal* 24: 462-467, 1992.

192. **Soma LR, Birks EK, Uboh CE, May L, Teleis D, and Martini J**. The effects of frusemide on racing times of Standardbred pacers. *Equine veterinary journal* 32: 334-340, 2000.

193. **Soma LR, and Uboh CE**. Review of furosemide in horse racing: its effects and regulation. *J Vet Pharmacol Ther* 21: 228-240, 1998.

194. **Somlyo AP, and Somlyo AV**. Signal transduction by G-proteins, rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. *J Physiol* 522 Pt 2: 177-185, 2000.

195. **Southgate KM, Mehta D, Izzat MB, Newby AC, and Angelini GD**. Increased secretion of basement membrane-degrading metalloproteinases in pig saphenous vein into carotid artery interposition grafts. *Arterioscler Thromb Vasc Biol* 19: 1640-1649, 1999.

196. **Stack A, Derksen FJ, Williams KJ, Robinson NE, and Jackson WF**. Lung Region and Racing Affect Mechanical Properties of Equine Pulmonary Microvasculature. *Journal of applied physiology* 2014.

197. **Stefanadis C, Karamanou M, and Androutsos G**. Michael Servetus (1511-1553) and the discovery of pulmonary circulation. *Hellenic journal of cardiology : HJC = Hellenike kardiologike epitheorese* 50: 373-378, 2009.

198. **Steibel JP, Poletto R, Coussens PM, and Rosa GJ**. A powerful and flexible linear mixed model framework for the analysis of relative quantification RT-PCR data. *Genomics* 94: 146-152, 2009.

199. **Stenmark KR, Davie N, Frid M, Gerasimovskaya E, and Das M**. Role of the adventitia in pulmonary vascular remodeling. *Physiology (Bethesda)* 21: 134-145, 2006.

200. **Stenmark KR, Frid MG, Yeager M, Li M, Riddle S, McKinsey T, and El Kasmi KC**. Targeting the adventitial microenvironment in pulmonary hypertension: A potential approach to therapy that considers epigenetic change. *Pulmonary circulation* 2: 3-14, 2012.

201. **Stenmark KR, Yeager ME, El Kasmi KC, Nozik-Grayck E, Gerasimovskaya EV, Li M, Riddle SR, and Frid MG**. The adventitia: essential regulator of vascular wall structure and function. *Annu Rev Physiol* 75: 23-47, 2013.

202. **Stickland MK, Lovering AT, and Eldridge MW**. Exercise-induced arteriovenous intrapulmonary shunting in dogs. *Am J Respir Crit Care Med* 176: 300-305, 2007.

203. **Stubenitsky R, Verdouw PD, and Duncker DJ**. Autonomic control of cardiovascular performance and whole body O2 delivery and utilization in swine during treadmill exercise. *Cardiovasc Res* 39: 459-474, 1998.

204. **Takahashi H, Soma S, Muramatsu M, Oka M, and Fukuchi Y**. Upregulation of ET-1 and its receptors and remodeling in small pulmonary veins under hypoxic conditions. *Am J Physiol Lung Cell Mol Physiol* 280: L1104-1114, 2001.

205. **Takahashi T, Hiraga A, Ohmura H, Kai M, and Jones JH**. Frequency of and risk factors for epistaxis associated with exercise-induced pulmonary hemorrhage in horses: 251,609 race starts (1992-1997). *J Am Vet Med Assoc* 218: 1462-1464, 2001.

206. **Tetens J, Derksen FJ, Stick JA, Lloyd JW, and Robinson NE**. Efficacy of prosthetic laryngoplasty with and without bilateral ventriculocordectomy as treatments for laryngeal hemiplegia in horses. *Am J Vet Res* 57: 1668-1673, 1996.

207. **Tian L, Lammers SR, Kao PH, Reusser M, Stenmark KR, Hunter KS, Qi HJ, and Shandas R**. Linked opening angle and histological and mechanical aspects of the proximal pulmonary arteries of healthy and pulmonary hypertensive rats and calves. *Am J Physiol Heart Circ Physiol* 301: H1810-1818, 2011.

208. **Toga H, Bansal V, and Raj JU**. Differential responses of ovine intrapulmonary arteries and veins to acetylcholine. *Respir Physiol* 104: 197-204, 1996.

209. **Tolsa JF, Gao Y, Sander FC, Souici AC, Moessinger A, and Raj JU**. Differential responses of newborn pulmonary arteries and veins to atrial and C-type natriuretic peptides. *Am J Physiol Heart Circ Physiol* 282: H273-280, 2002.

210. **Townsley MI**. Structure and composition of pulmonary arteries, capillaries, and veins. *Comprehensive Physiology* 2: 675-709, 2012.

211. **Tschanz SA, Burri PH, and Weibel ER**. A simple tool for stereological assessment of digital images: the STEPanizer. *Journal of microscopy* 243: 47-59, 2011.

212. **Tuchscherer HA, Vanderpool RR, and Chesler NC**. Pulmonary vascular remodeling in isolated mouse lungs: effects on pulsatile pressure-flow relationships. *Journal of biomechanics* 40: 993-1001, 2007.

213. **Tuder RM, Archer SL, Dorfmuller P, Erzurum SC, Guignabert C, Michelakis E, Rabinovitch M, Schermuly R, Stenmark KR, and Morrell NW**. Relevant issues in the pathology and pathobiology of pulmonary hypertension. *J Am Coll Cardiol* 62: D4-12, 2013.

214. **Tuder RM, Chacon M, Alger L, Wang J, Taraseviciene-Stewart L, Kasahara Y, Cool CD, Bishop AE, Geraci M, Semenza GL, Yacoub M, Polak JM, and Voelkel NF**. Expression of angiogenesis-related molecules in plexiform lesions in severe pulmonary hypertension: evidence for a process of disordered angiogenesis. *The Journal of pathology* 195: 367-374, 2001.

215. **Tulis DA, Unthank JL, and Prewitt RL**. Flow-induced arterial remodeling in rat mesenteric vasculature. *Am J Physiol* 274: H874-882, 1998.

216. **Vanhoutte PM**. Endothelial adrenoceptors. *J Cardiovasc Pharmacol* 38: 796-808, 2001.

217. **Vessieres E, Freidja ML, Loufrani L, Fassot C, and Henrion D**. Flow (shear stress)-mediated remodeling of resistance arteries in diabetes. *Vascul Pharmacol* 57: 173-178, 2012.

218. **Visse R, and Nagase H**. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ Res* 92: 827-839, 2003.

219. **Voelkel NF, Vandivier RW, and Tuder RM**. Vascular endothelial growth factor in the lung. *Am J Physiol Lung Cell Mol Physiol* 290: L209-221, 2006.

220. **Voynick BT, and Sweeney CR**. Exercised-induced pulmonary hemorrhage in polo and racing horses. *J Am Vet Med Assoc* 188: 301-302, 1986.

221. **Wallner B, Vogl C, Shukla P, Burgstaller JP, Druml T, and Brem G**. Identification of genetic variation on the horse y chromosome and the tracing of male founder lineages in modern breeds. *PLoS One* 8: e60015, 2013.

222. **Wallner K, Li C, Fishbein MC, Shah PK, and Sharifi BG**. Arterialization of human vein grafts is associated with tenascin-C expression. *J Am Coll Cardiol* 34: 871-875, 1999.

223. **Wallner K, Sharifi BG, Shah PK, Noguchi S, DeLeon H, and Wilcox JN**. Adventitial remodeling after angioplasty is associated with expression of tenascin mRNA by adventitial myofibroblasts. *J Am Coll Cardiol* 37: 655-661, 2001.

224. **Wan S, Yim AP, Johnson JL, Shukla N, Angelini GD, Smith FC, Dashwood MR, and Jeremy JY**. The endothelin 1A receptor antagonist BSF 302146 is a potent inhibitor of

neointimal and medial thickening in porcine saphenous vein-carotid artery interposition grafts. *J Thorac Cardiovasc Surg* 127: 1317-1322, 2004.

225. **Watts SW, Rondelli C, Thakali K, Li X, Uhal B, Pervaiz MH, Watson RE, and Fink GD**. Morphological and biochemical characterization of remodeling in aorta and vena cava of DOCA-salt hypertensive rats. *Am J Physiol Heart Circ Physiol* 292: H2438-2448, 2007.

226. **Weideman H, Schoeman SJ, Jordaan GF, and Kidd M**. Epistaxis related to exercise-induced pulmonary haemorrhage in south African Thoroughbreds. *Journal of the South African Veterinary Association* 74: 127-131, 2003.

227. **Weiler-Ravell D, Shupak A, Goldenberg I, Halpern P, Shoshani O, Hirschhorn G, and Margulis A**. Pulmonary oedema and haemoptysis induced by strenuous swimming. *Bmj* 311: 361-362, 1995.

228. **Wenzel D, Knies R, Matthey M, Klein AM, Welschoff J, Stolle V, Sasse P, Roll W, Breuer J, and Fleischmann BK**. beta(2)-adrenoceptor antagonist ICI 118,551 decreases pulmonary vascular tone in mice via a G(i/o) protein/nitric oxide-coupled pathway. *Hypertension* 54: 157-163, 2009.

229. **West JB, and Mathieu-Costello O**. Stress failure of pulmonary capillaries as a mechanism for exercise induced pulmonary haemorrhage in the horse. *Equine veterinary journal* 26: 441-447, 1994.

230. **West JB, Mathieu-Costello O, Jones JH, Birks EK, Logemann RB, Pascoe JR, and Tyler WS**. Stress failure of pulmonary capillaries in racehorses with exercise-induced pulmonary hemorrhage. *J Appl Physiol* 75: 1097-1109, 1993.

231. **Williams KJ, Derksen FJ, de Feijter-Rupp H, Pannirselvam RR, Steel CM, and Robinson NE**. Regional pulmonary veno-occlusion: a newly identified lesion of equine exercise-induced pulmonary hemorrhage. *Vet Pathol* 45: 316-326, 2008.

232. **Williams KJ, Derksen FJ, Defeijter-Rupp HL, and Robinson NE**. Repeated blood instillation into the airway of the horse does not cause pulmonary fibrosis. *Equine veterinary journal* 43: 354-358, 2011.

233. **Williams KJ, Robinson NE, Defeijter-Rupp H, Millerick-May M, Stack A, Hauptman J, and Derksen FJ**. Distribution of venous remodeling in exercise-induced pulmonary hemorrhage of horses follows reported blood flow distribution in the equine lung. *J Appl Physiol* 114: 869-878, 2013.

234. **Wolff RA, Malinowski RL, Heaton NS, Hullett DA, and Hoch JR**. Transforming growth factor-beta1 antisense treatment of rat vein grafts reduces the accumulation of collagen and increases the accumulation of h-caldesmon. *J Vasc Surg* 43: 1028-1036, 2006.

235. **Wong AP, Nili N, Jackson ZS, Qiang B, Leong-Poi H, Jaffe R, Raanani E, Connelly PW, Sparkes JD, and Strauss BH**. Expansive remodeling in venous bypass grafts: novel implications for vein graft disease. *Atherosclerosis* 196: 580-589, 2008.

236. **Wong MS, and Vanhoutte PM**. COX-mediated endothelium-dependent contractions: from the past to recent discoveries. *Acta pharmacologica Sinica* 31: 1095-1102, 2010.

237. **Wood JL, Newton JR, Chanter N, and Mumford JA**. Inflammatory airway disease, nasal discharge and respiratory infections in young British racehorses. *Equine veterinary journal* 37: 236-242, 2005.

238. **Younes M, Bshouty Z, and Ali J**. Longitudinal distribution of pulmonary vascular resistance with very high pulmonary blood flow. *Journal of applied physiology* 62: 344-358, 1987.

239. **Zellers TM, and Vanhoutte PM**. Heterogeneity of endothelium-dependent and independent responses among large and small porcine pulmonary arteries. *Pulmonary pharmacology* 2: 201-208, 1989.

240. **Zhang B, Zhou J, Li H, Liu Z, Chen A, and Zhao Q**. Comparison of graft patency between off-pump and on-pump coronary artery bypass grafting: an updated meta-analysis. *Ann Thorac Surg* 97: 1335-1341, 2014.

241. **Zhang J, Silva T, Yarovinsky T, Manes TD, Tavakoli S, Nie L, Tellides G, Pober JS, Bender JR, and Sadeghi MM**. VEGF blockade inhibits lymphocyte recruitment and ameliorates immune-mediated vascular remodeling. *Circ Res* 107: 408-417, 2010.

242. **Zhang RZ, Gashev AA, Zawieja DC, and Davis MJ**. Length-tension relationships of small arteries, veins, and lymphatics from the rat mesenteric microcirculation. *Am J Physiol Heart Circ Physiol* 292: H1943-1952, 2007.