Regional heterogeneity in the reactivity of equine small pulmonary blood vessels

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Stack A, Derksen FJ, Williams KJ, Robinson NE, Jackson WF. Regional heterogeneity in the reactivity of equine small pulmonary blood vessels. J Appl Physiol 120: 599–607, 2016. First published January 14, 2016; doi:10.1152/japplphysiol.00975.2015.—Regional differences in large equine pulmonary artery reactivity exist. It is not known if this heterogeneity extends into small vessels. The hypothesis that there is regional heterogeneity in small pulmonary artery and vein reactivity to sympathomimetics (phenylephrine and isoproterenol) and a parasympathomimetic (methacholine) was tested using wire myography on small vessels from caudodorsal (CD) and cranioventral (CV) lung of 12 horses [9 mares, 3 geldings, 8.67 ± 0.81 (age ± SE) yr, of various breeds that had never raced]. To study relaxation, vessels were precontracted with U46619 (10−6 M). Methacholine mechanism of action was investigated using L-nitroarginine methylster (L-NAME, 100 µM) and indomethacin (10 µM). Phenylephrine did not contract any vessels. Isoproterenol relaxed CD arteries more than CV arteries (maximum relaxation 28.18% and 48.67%; Log IC50 = −7.975 ± 0.1327 and −8.033 ± 0.1635 for CD and CV, respectively, P < 0.0001), but not veins. Methacholine caused contraction of CD arteries (maximum contraction 245.4%, Log EC50 = −6.475 ± 0.3341), and relaxation of CV arteries (maximum relaxation 40.14%, Log IC50 = −6.791 ± 0.1954) and all veins (maximum relaxation 50.62%, Log IC50 = −6.932 ± 0.1986) in a nonregion-dependent manner. L-NAME (n = 8, P < 0.0001) and indomethacin (n = 7, P < 0.0001) inhibited methacholine-induced relaxation of CV arteries, whereas indomethacin augmented CD artery contraction (n = 8, P < 0.0001). Our data demonstrate significant regional heterogeneity in small blood vessel reactivity when comparing the CD to the CV region of the equine lung.

NEW & NOTEWORTHY
Regional differences in large equine pulmonary artery reactivity exist however, it was not known if this heterogeneity extended into small vessels. The hypothesis that there is regional heterogeneity in small pulmonary vessel reactivity to sympathomimetics and a parasympathomimetic was tested using wire myography on small vessels from caudodorsal and cranioventral lung of horses, and results demonstrate significant regional heterogeneity in reactivity comparing caudodorsal with cranioventral vessels of the equine lung.

BLOOD FLOW DISTRIBUTION in the lung is determined by vascular anatomy, which is fixed, and variable factors including vascular reactivity to neurohumoral vasoactive substances (3, 6). Arterial and venous resistances to flow in the small vessels supplying and draining the capillaries are determined by vascular tone, and thus affect both local blood flow and pulmonary capillary pressure. To meet the physiologic demands of strenuous exercise, changes in pulmonary vascular tone play an important role in matching perfusion to ventilation to maximize aerobic capacity and gas exchange efficiency. In general, sympathetic nervous system activation associated with exercise results in both α- and β-adrenoreceptor activation in the pulmonary circulation (10, 35), whereas parasympathetic activity during exercise is diminished (22).

Some information is already known about pulmonary blood flow in the horse—for example, that blood flow is preferentially distributed to the caudodorsal lung in both the standing (8) and galloping horse (4). We recently reported that the pathology of exercise-induced pulmonary hemorrhage (EIPH) has a similar distribution (40), with lesion incidence and severity most marked in the caudodorsal lung. Stress failure of pulmonary capillaries is assumed to be a key event in EIPH pathogenesis. West et al. (38) described pulmonary capillary wall disruption in horses that had exercised on a treadmill and proposed that capillary rupture in exercising horses occurs as a result of high pulmonary capillary pressures (37), which are estimated to reach between 72 and 83 mmHg (18, 20). Further support for this theory was provided when the threshold for breaking strength of equine pulmonary capillaries was exceeded at 75 mmHg transmural pressure (5).

Our group has reported that regional differences in endothelial-dependent reactivity of large pulmonary arteries of the horse exist, and it is proposed that these differences might account in part for the gravity-independent flow distribution pattern (27). In large pulmonary arteries, 6 mm OD, methacholine-induced arteries from caudodorsal lung to relax and those from cranioventral lung to contract when vessel endothelium remained intact. Regional differences in responses of small pulmonary vessels to changes in the local concentration of neurohumoral substances, such as have been reported in larger caliber equine pulmonary vessels, could also be expected to modulate local vascular resistance and therefore exert varying effects on pulmonary capillary pressures in the lung.

Whether similar, regional differences in smaller caliber vessels in the horse lung exist has not been reported. However, we recently reported on regional heterogeneity in the mechanical properties of small pulmonary vessels (34). Differences in reactivity to various pharmacologic agents between pulmonary vessels of different diameter have been reported in other species including rats (15), pigs (41), and sheep (12), however, and could therefore be predicted to occur in the horse.

For these reasons, we investigated the hypothesis that regional differences in patterns of vascular reactivity to adrenergic and cholinergic agonists exist in small pulmonary arteries and veins of the horse. To test this hypothesis, we used wire...
myography to examine the reactivity of small pulmonary arteries and veins from unraced horses. Both adrenergic (phenylephrine and isoproterenol) and cholinergic (methacholine) agonists were tested.

MATERIALS AND METHODS

Animals. Nine horses (7 geldings and 2 mares), 8.33 ± 0.9 (average age ± SE; range 3 to 12, median age 9) yr, were used to determine regional patterns of reactivity to five drugs. Three additional horses (2 mares and 1 gelding), 9.67 ± 2.03 (average age ± SE; range 6 to 13, median age 10) yr, were used to investigate mechanisms of methacholine activity. Study horses were of various breeds [2 Quarter-horses, 2 Tennessee Walkers, 2 Thoroughbreds, 3 Arabian crosses, 3 grade horses (paint and Quarterhorses crosses)]. Horses were acquired by donation to the University and were evaluated for general and respiratory health by thorough physical examination. All horses were kept at the university farm for a number of weeks to months before euthanasia to ensure that they were not incubating respiratory disease upon presentation. Neither Thoroughbred horse had a race record on the Equibase official database, and both horses were deemed unfit for race training due to musculoskeletal unsoundness. Complete histories were compiled from the Equibase official database, and both horses were deemed unfit for racing.

Vessel normalization. Optimal passive tension values for small equine pulmonary arteries and veins have not been published. Therefore, the first 11 arteries harvested were tested 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 CaCl2, 1 MgCl2, 10 HEPES, 10 glucose (pH 7.4, 295 mosM). Vessels were allowed to develop a contraction for 2 min, the maximum T value reached was recorded, and the vessel was washed with PSS until T returned to baseline. After 5 min, 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 resting wall, T for veins was increased in 0.1 mN/mm increments.

The “peak” response of arteries tested (n = 11, from 4 horses) was achieved at an average set tension of 1.14 ± 0.18 mN/mm (mean ± SD) with upper and lower 95% confidence intervals of 1.009 and 1.271 mN/mm, respectively. These data were deemed adequately repeatable to use 1.1 mN/mm as a set tension value in subsequent experiments.

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 using minimal tension (0.1 mN/mm for arteries and 0.2 mN/mm for veins) at optimal tension (0.1 mN/mm for arteries, individual wall tension for veins) conditions.

Vessel wake-up. Once normalized, vessels were allowed a 40-min equilibration period (based on manufacturer’s recommendation and experimental observation) at their optimal T. Then vessels underwent a wake-up procedure consisting of two challenges with 60 mM K+ PSS with a 5-min interval. Failure to maintain a sustained, repeatable contraction at this point resulted in rejection of the vessel for further experiments. Eighty percent of all vessels that were mounted on the myograph went through the wake-up procedure, and of these, 83.65% met the acceptance criterion outlined above and were deemed eligible for further testing.

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 was performed. Concentration ranges were initially established based on the literature from other species and then confirmed in equine vessels during preliminary experiments. U46619, methacholine, and isoproterenol concentrations ranged from 1 × 10⁻⁶ to 3 × 10⁻⁴ M.

To evaluate responses to vasodilator agents (methacholine and isoproterenol), all vessels were first exposed to 10⁻⁶ M U46619, a concentration that was confirmed as reliably producing maximal vasoconstriction in both arteries and veins during preliminary experiments. U46619, methacholine, and isoproterenol concentrations ranged from 1 × 10⁻⁶ to 3 × 10⁻⁴ M.

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 four 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. Thirty minutes after vessel wake-up, vessels were precontracted with 10⁻⁶ M U46619, and methacholine CCRCs were performed on all vessels. After washing, vessels were incubated with either l-NMMA (10⁻⁴ M) or indomethacin (10⁻⁵ M) for 30 min, and the methacholine CCRCs were repeated. After washing, all vessels were incubated with l-NMMA and indomethacin for 30 min and a
third methacholine CCRC was performed in the presence of both inhibitors.

Vessel fixation. After vessel reactivity 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 min.

Immunohistochemistry. After washing with PBS, immunofluorescent staining for endothelium (CD-31) was carried out on the whole-mount 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, which were incubated overnight at 4°C. After blocking (with 5% normal goat serum in a 1% saponin in PBS solution) for 1 h, Alexa Fluor 488-conjugated AffiniPure goat anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) (1:100) was applied for 1 h. Vessels were then mounted on slides under PBS.

Endothelial imaging. Endothelium was examined using epifluorescent microscopy. Each vessel was photographed at ×20 magnification, and depending on vessel surface area, between 2 and 7 images per vessel were acquired and saved. Endothelium-covered regions were outlined using imaging software (30), and total endothelium-covered area was expressed as a percentage of the total tissue area in an image.

Vessel histology. After fixation or imaging, vessels were removed from the Petri dish or slide and placed in Histogel (American Laboratories, West Grove, PA) (1:100) was applied for 1 h. Vessels were then mounted on slides under PBS solution) for 1 h, Alexa Fluor 488-conjugated AffiniPure goat anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) (1:100) was applied for 1 h. Vessels were then mounted on slides under PBS.

Materials. U46619 was acquired from Tocris Bioscience (Minneapolis, MN). Acetyl-β-methylcholine chloride, phenylephrine hydrochloride, isoproterenol hydrochloride, N-ω-nitro-arginine methyl ester hydrochloride (lNAME), and indomethacin were acquired from Sigma-Aldrich (St. Louis, MO). U46619 was 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 (U46619 and phenylephrine) were expressed as a percentage of the mean of that vessel’s maximal contractions in response to 60 mM KCl-PSS during vessel wake-up. Once a stable contraction to 10−6 M U46619 was established, all responses to vasodilator agents (methacholine and isoproterenol) were expressed as a percentage of that contraction.

Cumulative concentration response curves were fit to a log(agonist) vs. response sigmoidal curve \( Y = bottom + [(top - bottom)/(1+10^{LogEC_{50}-X}*Hill Slope}) \], and curve fits were then compared between arteries and veins and between lung regions (GraphPad Prism 6, GraphPad Software, La Jolla, CA). EC_{50} 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 ± SE, and \( P < 0.05 \) was 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) 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.

Histology. Fifty-one 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. Two vessels that were dissected as veins were identified as arteries using histology, and data were included in the artery dataset. One artery could not be confirmed as such because of 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.

Vessel dimensions. Diameter of arteries and veins under minimal tension of 0.1 mN/mm was 169.9 ± 10.91 (mean ± SE) and 128.3 ± 9.0 µm, respectively. Under optimal tension, arterial diameter was 367 ± 16.23 (mean ± SE) µm, whereas venous diameter was 205.8 ± 20.86 (mean ± SE) µm. By using the Law of Laplace to convert wall tension (in mmHg) 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 ± SE) mmHg in arteries and veins, respectively.

U46619. U46619 caused concentration-dependent contraction in both arteries (\( n = 16 \)) and veins (\( n = 15 \)) (Fig. 2A), with the greatest response observed in veins. The maximum response in 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 U46619 than pulmonary arteries with \( \log EC_{50} \) values of −7.011 ± 0.096 M and −6.563 ± 0.05 M (\( P < 0.0001 \)) in veins and arteries, respectively. When evaluated by region, caudodorsal (\( n = 7 \)) and cranioventral (\( n = 9 \)) arteries and veins (\( n = 9 \)) did not differ in their responses to U46619 (Fig. 1B; \( P = 0.25 \)), whereas a regional effect was observed in pulmonary veins with cranioventral (\( n = 8 \)) veins demonstrating enhanced sensitivity compared with caudodorsal veins (\( n = 7 \); Fig. 1C; \( P < 0.0001 \)).

Phenylephrine. Neither pulmonary arteries (\( n = 10 \)) nor pulmonary veins (\( n = 9 \)) contracted in response to \( 10^{-5} \) M phenylephrine (Fig. 2).

Isoproterenol. In vessels precontracted with U46619, isoproterenol caused a concentration-dependent relaxation in pulmonary arteries (\( n = 11 \)), whereas 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 (\( P < 0.0001 \); Fig. 3A) with caudodorsal arteries demonstrating enhanced relaxation (relaxation 28.18 ± 3.74% and 48.67 ± 3.09% of maximum and \( \log IC_{50} \) ± SE = −7.975 ± 0.1327 and −8.033 ± 0.1635 in CD and CV arteries, respectively). There was no regional difference in the response of veins to isoproterenol (Fig. 3B).

Methacholine. The response of arteries to methacholine varied by region (\( n = 16 \)). All cranioventral arteries (\( n = 8 \)) demonstrated a concentration-dependent relaxation (maximum relaxation 40.14%, \( \log IC_{50} \) ± SE = −6.791 ± 0.1954), whereas
all caudodorsal arteries \( (n = 8) \) contracted, also in a concentration-dependent manner (Fig. 4A; maximum contraction 245.4\%, \( \text{LogEC}_{50} = -6.475 \pm 0.3341 \)). Methacholine caused a concentration-dependent relaxation in pulmonary veins precontracted with U46619 (10\(^{-6}\) M; \( n = 13 \), maximum relaxation 50.62\%, \( \text{LogIC}_{50} = -6.932 \pm 0.1986 \)) but the response to methacholine did not differ by region (\( n = 6 \) and \( n = 7 \) for CD and CV veins, respectively; \( P = 0.59 \); Fig. 4B).

Mechanisms of methacholine reactivity. Preincubation of caudodorsal pulmonary arteries (\( n = 7 \)) with \( l^{-}\)-NAME did not affect their response (contraction) to methacholine (\( P = 0.7 \); Fig. 5A). Preincubation with indomethacin, however, augmented the contraction of CD arteries (\( n = 8 \)) compared with methacholine alone (\( P < 0.0001 \); Fig. 5B). Preincubation of CD arteries (\( n = 13 \)) with both \( l^{-}\)-NAME and indomethacin also resulted in an augmented contraction (Fig. 5C) but the concentration-response relationship did not differ from vessels incubated with indomethacin alone (\( P = 0.79 \)).

Preincubation of CV pulmonary arteries (\( n = 8 \)) with \( l^{-}\)-NAME inhibited methacholine-induced relaxation (\( P < 0.0001 \); Fig. 5D). Preincubation with indomethacin also resulted in reduced relaxation of CV arteries (\( n = 7 \); Fig. 5E). Preincubation of CV arteries (\( n = 15 \)) with both \( l^{-}\)-NAME and indomethacin induced a mild contraction and abolished relax-
regions (vessels, respectively, and these values did not differ between veins relax in a concentration-dependent manner, regardless of region (B)). Precontracted pulmonary vascular smooth muscle in the horse relax, whereas those from cranioventral lung regions show concentration-dependent constriction occurred in CD arteries (A); precontracted pulmonary veins relax in a concentration-dependent manner, regardless of region (B).

Concentration-dependent relaxation occurs in CV arteries, and concentration-dependent constriction occurred in CD arteries (A); precontracted pulmonary veins relax in a concentration-dependent manner, regardless of region (B).

Fig. 4. Cumulative concentration response curves for methacholine for CD and 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); precontracted pulmonary veins relax in a concentration-dependent manner, regardless of region (B).

Regional differences in responses of pulmonary arteries to thromomimetics. Specifically, the selective α1- and nonselective 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 (33). These concentrations (~1.5 × 10⁻⁷ M) fall within the concentration range over which isoproterenol, an adrenergic agonist, was tested.

Before evaluation of vessel reactivity, a normalization procedure was performed to determine the degree of smooth muscle stretch that would result in maximal force development for subsequent experiments (1). Optimal passive wall tension of arteries was significantly larger than that of veins. In horses, mean in vivo resting pulmonary arterial pressures are ~30 mmHg (14, 18, 32), whereas in vivo pulmonary artery wedge pressures (a proxy for venous pressures) range from 13.4 to 18 mmHg (14, 18, 32). Conversion of optimal wall tension to equivalent pressure values demonstrated that these studies were conducted at physiologically relevant (resting) tensions (24.2 and 14.1 mmHg in arteries and veins, respectively).

The thromboxane A₂ analog U46619 was selected as a tool for subsequent experiments in which preexisting tone was necessary to study another vasoactive agent. U46619 caused contractions in both pulmonary arteries and veins, and pulmonary veins were more sensitive to U46619 than arteries. Cranioventral veins were also more sensitive to this agent than their caudal counterparts. Enhanced sensitivity to U46619 in pulmonary veins compared with pulmonary arteries has also been reported in other species, including sheep, dogs, and guinea pigs (2, 12, 31), but the role of thromboxane in

The present study was designed to investigate whether regional differences in small pulmonary vessel reactivity exist in the horse. Such differences in large (6 mm OD) equine pulmonary arteries have already been reported (27). We found regional differences in responses of pulmonary arteries to isoproterenol and methacholine and in responses of veins to U46619. We also performed further investigations into mechanisms of methacholine reactivity in pulmonary arteries based on observed differences. These findings support our hypothesis. The patterns of reactivity to methacholine observed in the small vessels in these studies differ from those observed in larger vessels (27) (large equine pulmonary arteries from caudal lung relax, whereas those from cranioventral lung contract in response to methacholine; and small equine caudal lung arteries contract, whereas small cranioventral arteries relax in response to methacholine), indicating that information gleaned from larger pulmonary vessels cannot be extrapolated to smaller vessels, even in the same species.

Based on an extensive literature search using PubMedCentral (NIH/NLM) performed at the time of writing (November 2015), no studies with similar outcomes have been published; therefore this study reports for the first time in any species that regional differences in reactivity exist in smaller-caliber pulmonary arteries and veins. The vessels of interest in this investigation are small (100–400 μm OD) pulmonary arteries and veins. We recently reported on regional heterogeneity in mechanical characteristics of equine pulmonary vessels of this caliber and suggest that these anatomical differences are related to the preferential distribution of blood flow in the equine lung to caudal regions (34). Our present investigation extends these studies and shows that there are also significant regional differences in reactivity of small vessels in the equine lung.

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, whereas parasympathetic activity is diminished (22, 35). 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 (10, 35). For these reasons, we focused our investigations onto the regional reactivity of small pulmonary arteries and veins of the horse to sympathetic and parasympathomimetics. Specifically, the selective α1- and nonselective 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 (33). These concentrations (~1.5 × 10⁻⁷ M) fall within the concentration range over which isoproterenol, an adrenergic agonist, was tested.

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regulation of vascular tone during exercise, however, is considered minimal (26).

Although large equine pulmonary arteries (1.5–4 mm in diameter)(16) and the largest pulmonary veins (7) contract in response to phenylephrine, phenylephrine did not cause contraction in either arteries or veins in the present study, suggesting an absence of the target α1-receptor in smaller caliber vessels. This observation is not unique to the horse. Small pulmonary arteries (100–300 μm in diameter) of the rat do not respond reliably to norepinephrine (15), and the response of ovine pulmonary arteries to norepinephrine is attenuated with decreasing vessel diameter (12).

We also found that precontracted equine pulmonary arteries relaxed in response to the β-adrenergic receptor agonist isoproterenol, whereas pulmonary veins failed to respond. Furthermore, isoproterenol-induced relaxation was of greater mag-

Fig. 5. 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 after preincubation with l-NAME (A and D), indomethacin (B and E), and l-NAME and indomethacin (C and F). Values are means ± SE. Preincubation 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 preincubation augments CD artery constriction, and partially inhibits CV artery relaxation (B and E, respectively). Preincubation 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).
nitude in the caudodorsal than cranioventral arteries. This is noteworthy, because, based on an extensive literature search using PubMedCentral (NIH/NLM) performed at the time of writing (November 2015), there are no other reports of regional differences in distribution of beta-adrenoreceptors in the pulmonary vasculature of any species.

In equine small pulmonary vessels, β-adrenoreceptor-mediated vasodilation could predominate during exercise (as there was no evidence of α₁-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. Such a regional increase in intravascular pressures could result in increased capillary stress failure (37, 38), extravasation of blood into the pulmonary interstitium and airways, and consequential pathologic changes of EIPH, including venous remodeling, hemosiderin accumulation, and fibrosis (40). We speculate that this sequence of events could explain, at least in part, the regional caudodorsal distribution of EIPH in the horse lung.

We found that small, precontracted pulmonary veins of the horse relaxed in response to methacholine. Pulmonary arteries, on the other hand, demonstrated opposite effects depending on lung region. Arteries from caudodorsal lung contracted whereas those from cranioventral lung relaxed in response to methacholine.

In general, binding of acetylcholine to muscarinic receptors on the endothelium, or on the smooth muscle of blood vessels, causes vasodilation and vasoconstriction, respectively (22, 35). Whether equine blood vessels of this caliber are directly innervated by the parasympathetic nervous system has not been reported; 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 (27) and in the pig (29). However, both studies reported on vessels that were much larger (4–6 mm OD) than those evaluated in the present study. In porcine pulmonary arteries, more pronounced relaxation in response to acetylcholine was observed in dorsal vessels compared with those from ventral lung (29), and large equine pulmonary arteries from caudodorsal lung also relax, whereas those from cranioventral lung contract in response to methacholine (27). We observed a different pattern of reactivity to methacholine: caudodorsal arteries contracted, whereas cranioventral arteries relaxed. The contrasting pattern observed in equine small pulmonary arteries is of particular interest when considered in the context of EIPH and EIPH lesion distribution. If parasympathetic outflow contributes to 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 and then diminished parasympathetic activity during exercise (22) could result in reduced arterial tone, in the 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 with capillaries in other lung regions, perhaps contributing to stress failure of capillaries in this region (37, 38) and, ultimately, region-specific EIPH pathologic changes (40). However, when extrapolating these data to in vivo conditions, it is noted that all vessels in these studies were precontracted, and responses of pulmonary vessels to acetylcholine can vary depending on whether vascular tone is present (2).

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. We observed that L-NAME did not affect the response of caudodorsal vessels to methacholine, whereas indomethacin preincubation resulted in augmented contraction. These data indicate that some prostanoid-mediated suppression of contraction was occurring in caudodorsal arteries but was masked by the magnitude of the contraction. In cranioventral arteries, L-NAME attenuated methacholine-induced relaxation, as did indomethacin. Coincubation 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 to muscarinic receptor agonists.

Nitric oxide contributes to maintenance of basal pulmonary vasomotor tone in the horse (17). 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 (13, 24). Furthermore, administration of L-NAME to horses at rest results in significant increases in pulmonary arterial, capillary, and venous pressures (19). 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

Fig. 6. Fluorescent staining of CD-31 on endothelial surface of a small equine pulmonary artery. Regions of intact endothelium can be discerned from endothelium-denuded, unstained regions (indicated by arrowheads). Scale bar = 100 μm.
effect of modulation 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 (25). The effect of cyclooxygenase inhibition on pulmonary vasculature as an off-target effect of nonsteroidal anti-inflammatory medications may merit future consideration.

Data in this study were acquired from horses that had not raced, although the authors recognize that the horses may have exercised strenuously at times in their life. Incomplete exercise histories on all horses used in this study is acknowledged as a weakness of the study. However, according to racing databases, the Thoroughbreds had never raced and the remaining horses are breeds that are not typically used for racing and other strenuous competition also associated with EIPH (such as 3-day eventing and polo). Furthermore, no evidence of gross lesions typical of EIPH (40) were observed on the pleural surfaces of lungs used, and for these reasons it is assumed for the purposes of this study that these horses’ lungs were unaffected by significant EIPH-associated pathology. Horses that have trained and raced have remodeled pulmonary veins (40), and racing is associated with increased wall stiffness of caudodorsal veins (34). It is reported in pigs that remodeling of pulmonary vessels and associated structural changes are associated with alterations in vessel reactivity (11). Therefore, remodeled pulmonary veins such as those seen in EIPH-affected lung (39) may react differently than vessels used in this study. Furthermore, exercise training has been demonstrated to improve pulmonary artery dilation in response to acetylcholine (9) 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.

The findings of this study support our hypothesis that regional differences in patterns of vascular reactivity to adrenergic and cholinergic agonists exist in small pulmonary arteries and veins of the horse. If these results can be extrapolated to the exercising horse and are considered in the context of preferential blood flow distribution to caudodorsal lung—the pattern of these regional differences may shed further light on the role of the small pulmonary veins and arteries in determining local vascular pressures and how these can differ considerably based on lung region.

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AUTHOR CONTRIBUTIONS


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