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Equine Cardiology

Renin-Angiotensin-Aldosterone System Profiling in Horses Before and After Exercise

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ABSTRACT

Background: The impact of exercise on the classical and alternative renin-angiotensin-aldosterone system (RAAS) pathways has not been studied in horses.

Hypothesis: We hypothesized that exercise would activate both RAAS pathways and that endurance exercise would cause more activation of the classical pathway compared to short-duration, high-intensity exercise in horses.

Animals: Twenty-five horses (21 client-owned and 4 research) were included in 4 exercise groups (10 Arabians, 50-mile ride [A-E]; 4 thoroughbreds, 1-mile treadmill exercise [TB-TM]; 5 thoroughbreds, 1–1/16th-mile race [TB-R]; and 6 quarter horses 330-500-yard race [QH-R]).

Methods: Blood was collected before and after exercise. Equilibrium analysis was performed to measure serum RAAS metabolites and enzyme activities. The components of the RAAS pathways were compared before and after exercise. Post/pre-exercise ratios for each variable were compared among exercise groups. Data were reported as median (first, third quartiles; pre vs. post) and $p < 0.05$ was considered significant.

Results: Exercise increased classical RAAS metabolites (pmol/L; angiotensin I, 2.5 [2.5, 2.5] vs. 8.2 [2.5, 19.0]; angiotensin II, 10.2 [6.0, 21.9] vs. 53.0 [37.4, 95.8]; aldosterone, 83.8 [53.4, 149.5] vs. 170.6 [112.2, 251.7]); alternative RAAS metabolites (pmol/L; angiotensin 1–7, 1.5 [1.5, 1.5] vs. 5.1 [1.5, 12.5]; angiotensin 1–5, 2.5 [2.5, 7.3] vs. 14.9 [9.0, 25.4]) and angiotensin-converting enzyme-2 activity (ng/mL; 16.6 [13.9, 20.4] vs. 25.2 [20.2, 33.0]; $p < 0.001$) for all horses. Angiotensin 1–7 ratios were higher for TB-R compared with TB-TM and A-E ($p < 0.001$).

Conclusions and Clinical Importance: Both classical and alternative RAAS pathways increase after exercise in horses.

1 | Introduction

The renin-angiotensin-aldosterone system (RAAS) plays a pivotal role in the regulation of blood pressure and fluid homeostasis by influencing sodium and water reabsorption as well as vascular tone. Activation of this neurohormonal axis leads to the cleavage of pro-renin and renin release, which catalyzes the formation of angiotensin I from angiotensinogen. Angiotensin-converting enzyme (ACE) cleaves angiotensin I to angiotensin II, which is the final effector of classical effects,

Abbreviations: AA2, aldosterone/angiotensin II ratio; ACE, angiotensin-converting enzyme; AE, Arabian endurance horse; Ang 1–5, angiotensin 1–5; Ang 1–7, angiotensin 1–7; Ang 1–9, angiotensin 1–9; Ang 2–10, angiotensin 2–10; Ang 2–7, angiotensin 2–7; Ang 3–7, angiotensin 3–7; Ang I, angiotensin I; Ang II, angiotensin II; Ang III, angiotensin III; Ang IV, angiotensin IV; NEP, neprilysin; PRA, plasma renin activity; QH-R, quarter horse racing; RAAS, renin-angiotensin-aldosterone system; TB-R, thoroughbred racing; TB-TM, thoroughbred treadmill.

including vasoconstriction and the release of arginine vasopressin and aldosterone to modulate water and sodium retention, respectively [1, 2]. Further cleavage of the C terminus of angiotensin II by aminopeptidases leads to the formation of angiotensin III and IV [3]. Angiotensin III causes vasoconstriction and aldosterone release, and angiotensin IV plays a role in cognitive function [4, 5]. The counter-regulatory arm of the RAAS is mediated by several enzymes including angiotensin-converting enzyme 2 (ACE2), neprilysin (NEP), prolyl-carboxy-peptidase, and prolyl-endo-peptidase [6–8]. Products of this alternative RAAS pathway include angiotensin 1–7, angiotensin 1–9, and angiotensin 1–5 [3, 6]. Angiotensin 1–7 is the primary effector of this pathway that counterbalances the classical arm of the RAAS through its vasodilatory and natriuretic actions [9].

Activation of the RAAS is an appropriate response to physiologic stressors, facilitating adaptation to blood volume or blood pressure abnormalities. Disease states cause chronic classical RAAS upregulation that can be maladaptive. The impact of chronic activation of the classical RAAS pathway in congestive heart failure, systemic hypertension, and chronic kidney disease has been well documented in humans and animals, but much less is known about the counter-regulatory, alternative pathway in both health and disease [2, 10, 11].

The pathogenesis of metabolic complications associated with exercise in horses (e.g., exercise-induced pulmonary hemorrhage) is elusive. Considering that some RAAS components are located in lung tissue, it is possible that the RAAS is involved in exercise-related disease or the response to pulmonary stressors in horses. In one study, racing thoroughbreds with more severe exercise-induced pulmonary hemorrhage had higher ACE activity in their circulation [12]. Recent studies have documented that exercise in humans is associated with activation of the alternative RAAS pathway, resulting in an increase in circulating angiotensin 1–7 [13–15]. Exercise in horses causes classical RAAS activation, leading to an increase in plasma renin activity (PRA), aldosterone, and angiotensin II, but the impact of exercise on alternative angiotensin metabolites has not been studied in horses [16–18]. Additionally, the effect of exercise type on the comprehensive RAAS profile has not been evaluated.

Our objective was to evaluate the impact of exercise on both the classical and alternative pathways of the RAAS in horses performing different exercise regimens. We hypothesized that the alternative and classical RAAS pathways would be activated by exercise in horses and that endurance exercise would result in more activation of the classical arm compared to short, intensive exercise.

2 | Methods

2.1 | Horses

Twenty-five clinically healthy horses that were exercised at distances ranging from 330 yards to 50 miles were included in the study. Four horses were owned by the University of Florida as part of a research herd, and the remaining 21 were client-owned. All horses were classified into one of four exercise groups: (a) four conditioned thoroughbreds (2 mares, 2 geldings; ages 3–9 years) completed a standard exercise test (2-min mile) with a warm-up of 0.6 km at 4 m/s, then 1.6 km at 13.5 m/s, and a cool down of 0.6 km at 4 m/s on a high-speed treadmill (no incline) [TB-TM]; (b) 10 Arabians (6 mares, 2 geldings; ages 6–18 years) completed a 50-mile (80 km) endurance ride [A-E]; (c) five thoroughbreds (2 mares, 3 geldings; ages 4–7 years) raced 1 and 1/16th miles (1700 m) [TB-R]; and (d) six quarter horses (2 mares, 4 geldings; aged 3 years) raced 330–500 yards (302–457 m) [QH-R]. Blood samples were obtained by jugular venipuncture immediately before and within 5 min after exercise completion. After centrifugation (3500 g for 10 min), serum was collected and stored at −80°C before shipment on dry ice to the laboratory for analysis. Angiotensin metabolite quantification and enzyme metabolite analysis were completed within 60 and 130 days of blood collection, respectively.

2.2 | Equilibrium Analysis of RAAS Components

Liquid chromatography-mass spectrometry/mass spectroscopy (LC–MS/MS) was performed at a service provider laboratory (Attoquant Diagnostics, Vienna, Austria) using methodology previously validated in humans, dogs and cats [6, 10, 19]. The equilibrium concentration of 10 different angiotensin metabolites (Ang I, angiotensin I; Ang II, angiotensin II; Ang 1–9, angiotensin 1–9; Ang 2–10, angiotensin 2–10; Ang 1–7, angiotensin 1–7; Ang 1–5, angiotensin 1–5; Ang 2–7, angiotensin 2–7; Ang 3–7, angiotensin 3–7; Ang III, angiotensin III; Ang IV, angiotensin IV), and aldosterone were quantified. Briefly, samples were equilibrated for 1 h at 37°C, stabilized, and spiked with stable isotope-labeled internal standards for angiotensins and a deuterated internal standard for aldosterone, and analytes were extracted using C18-based solid-phase extraction. Internal standards were used to correct for analyte recovery across the sample preparation procedure in each individual sample. Angiotensin I and angiotensin II were summed as a marker for PRA. The ratio of aldosterone to angiotensin II (AA2) was calculated to characterize adrenal sensitivity to aldosterone release from angiotensin II stimulation. The lower limits of detection (pmol/L) for the angiotensin metabolites were as follows: Ang I, 5.0; Ang II, 1.0; Ang 1–9, 4.0; Ang1–7, 3.0; Ang 1–5, 5.0; Ang 2–10, 2.0; Ang III, 5.0; Ang IV, 2.0; Ang 2–7, 2.0; and Ang 3–7, 3.0. Metabolite concentrations below the lower limit of quantification were entered as half the lower limit of quantification for statistical comparisons. The LC–MS/MS-based assay was validated in compliance with the European Medicines Agency guideline “Guideline on bioanalytical method validation”. The LC–MS/MS-based assay has an accuracy and precision both within a run and between runs within 15% of the nominal value for all peptides and within 20% of the nominal value for the lower limit of quantitation. Because the amino acid sequence of equine angiotensins is identical to human, canine, feline, and rodent angiotensins, it was possible to make use of the validated LC–MS/MS assay with internal controls in the sample to account for changes in a matrix that might occur between species.

For NEP and ACE2 activity measurement, samples were spiked with an excess of either Ang I or Ang II and incubated at 37°C for 60 min in the presence and absence of an NEP inhibitor and

ACE2 inhibitor, respectively. The Ang 1–7 formation rate was calculated in (pmol/L)/h. The Ang 1–7 formation rate was subsequently converted to ng/ml, using the linear calibration obtained from a standard curve of recombinant human ACE2 or NEP, respectively.

For ACE activity measurement, samples were spiked with excess Ang I and incubated at 37°C for 60 min in the presence and absence of an ACE inhibitor. The Ang II formation rate was calculated in (pmol/L)/h. The Ang II formation rate was subsequently converted to ng/ml using the linear calibration obtained from a standard curve of recombinant human ACE.

The following metabolites and enzymes are considered part of the classical RAAS pathway: Ang I, Ang II, Ang III, Ang IV, Ang 2–10, Ang 2–7, ACE, and aldosterone. The following metabolites and enzymes are considered part of the alternative RAAS pathway: Ang 1–9, Ang 1–7, Ang 1–5, ACE2, and NEP. The value of each RAAS component after exercise to before exercise (post/pre) was calculated to quantify the magnitude of change as a result of exercise.

2.3 | Statistical Analysis

Concentrations of RAAS metabolites and enzyme activities were calculated and reported as median (first and third quartiles). The distribution of variables for RAAS concentrations was not normally distributed. The null hypothesis that paired RAAS measurements were not different before and after exercise for all horses in the study was tested by using the non-parametric Wilcoxon signed-rank test. In addition, the null hypothesis that RAAS concentration ratio values (post/pre-exercise) were not different among AE, TB-R, QH-R, and TB-TM exercise groups was tested by using the non-parametric Kruskal-Wallis test followed by Dunn's test for multiple comparisons. Values of $p < 0.05$ were considered significant. All analyses were performed using commercial software (Statistix 10. Analytical Software. Tallahassee, FL).

2.4 | Results

2.4.1 | Effect of Exercise

With the exception of Ang II and Ang 1–5, pre-exercise results for angiotensin metabolites were below the limit of detection in the majority of horses (Table 1). Exercise significantly increased Ang I, Ang II, Ang 1–7, Ang 1–5, aldosterone, PRA, and ACE2 activity, and significantly decreased AA2 when all horses ($n = 25$) were evaluated as a group ($p < 0.001$). The renin-angiotensin fingerprint representing the equilibrium concentrations of analytes is depicted in Figure 1. Significant increases of selected analytes among different exercise groups were noted for A-E (Ang I, Ang II, Ang 1–5, PRA, ACE2; all $p < 0.031$) and QH-R (Ang II, Ang 1–5, aldosterone, PRA, and ACE2; all $p = 0.03$) but not for TB-R or TB-TM. The AA2 was significantly lower after exercise in A-E ($p = 0.003$) but not for the other exercise groups. Changes in the activities of ACE and NEP were not significant, but ACE 2 activity significantly increased after exercise in A-E ($p = 0.002$) and QH-R ($p = 0.03$) groups (Figure 2).

2.4.2 | Comparison of the Effect of Exercise Among Different Groups

Significant differences among exercise groups were found for Ang II, Ang 1–7, Ang 2–7, Ang 1–5, AA2, PRA, and ACE2 (Table 2).

2.4.2.1 | Significant Differences Between A-E and Other Exercise Groups. The ratios of post/pre-exercise for Ang II and Ang 1–5 were significantly higher, and AA2 was significantly lower for A-E compared to TB-TM ($p \leq 0.03$). The post/pre-exercise ratio for Ang 1–7 was significantly lower for A-E compared with TB-R ($p < 0.001$).

2.4.2.2 | Significant Differences Between TB-R and Other Exercise Groups. The ratio of post/pre-exercise for Ang II, Ang 1–7, Ang 2–7, PRA, and ACE-2 was significantly higher and AA2 was significantly lower for TB-R compared with TB-TM horses ($p \leq 0.03$).

3 | Discussion

In our study of healthy horses, exercise stimulated both the classical and alternative pathways of the RAAS. In support of our hypothesis, several classical RAAS components (PRA, Ang 1, and Ang II) were significantly increased after exercise in endurance horses. Plasma renin activity, Ang II, and aldosterone also increased significantly after short, intense exercise in the QH-R group, but not in the TB-R and TB-TM groups. Previous studies in endurance horses also documented increases in classical RAAS components after exercise, with more RAAS stimulation in unsuccessful horses that developed more severe dehydration [16]. We found a higher increase in Ang II relative to the increase in aldosterone, which was reflected in the lower AA2 after exercise in endurance horses, but the clinical relevance of this finding is not known. Concurrent measurement of laboratory results to document the post-exercise hydration status of the horses in our study may have provided additional useful information to interpret the differences in RAAS stimulation between groups.

In a previous treadmill study, a linear correlation between exercise intensity and both PRA and aldosterone production was noted in maximally exercising horses [17]. Finding higher results for some RAAS metabolites for TB-R compared with TB-TM in our study is consistent with the previously published relationship because the TB-TM horses were exercised submaximally. Notably, we also found significant increases in the major enzyme of the alternative RAAS pathway (ACE2) and its metabolite (Ang 1–7) after exercise in all horses, with the highest increase in TB-R compared with TB-TM (ACE2 and Ang 1–7) and A-E (Ang 1–7). These findings indicate that exercise in horses is characterized by global RAAS activation, which might be most pronounced with the type of acute and intense exercise associated with track racing over one mile. Interestingly, the QH-R group did not have significantly higher results for RAAS activation compared to the other exercise groups even though the intensity of exercise in this group was likely similar to that achieved by TB-R. Breed differences might have affected the findings separately from the exercise type because distinct breeds are used for specific equine sports. Additional

TABLE 1 | Paired comparisons of RAAS concentrations in 25 healthy horses pre-and post-exercise*.

RAAS		All $n=25$	A-E $n=10$	TB-R $n=5$	QH-R $n=6$	TB-TM $n=4$
Ang I (pmol/L)	Pre	2.5 (2.5, 2.5)	2.5 (2.5, 2.5)	2.5 (2.5, 2.5)	2.5 (2.5, 4.0)	2.5 (2.5, 4.5)
	Post	8.2 (2.5, 19.0)	7.4 (2.5, 31.9)	18.7 (6.1, 54.9)	8.3 (2.5, 12.1)	4.7 (2.5, 9.6)
	p	<0.001	0.03	0.12	0.12	ND
Ang II (pmol/L)	Pre	10.2 (6.0, 21.9)	7.1 (2.9, 14.1)	12.4 (5.2, 16.0)	11.0 (9.1, 24.8)	25.3 (9.1, 39.4)
	Post	53.0 (37.4, 95.8)	46.5 (29.5, 101.9)	110.7 (65.0, 246.8)	53.7 (41.8, 83.6)	28.9 (8.1, 51.5)
	p	<0.001	<0.001	0.06	0.03	0.25
Ang III (pmol/L)	Pre	2.5 (2.5, 2.5)	2.5 (2.5, 2.5)	2.5 (2.5, 2.5)	2.5 (2.5, 2.5)	2.5 (2.5, 2.5)
	Post	2.5 (2.5, 2.5)	2.5 (2.5, 2.5)	2.5 (2.5, 13.1)	2.5 (2.5, 2.5)	2.5 (2.5, 2.5)
	p	ND	1.00	ND	1.00	1.00
Ang IV (pmol/L)	Pre	1.0 (1.0, 1.0)	1.0 (1.0, 1.0)	1.0 (1.0, 1.0)	1.0 (1.0, 1.0)	1.0 (1.0, 1.0)
	Post	1.0 (1.0, 1.0)	1.0 (1.0, 1.6)	1.0 (1.0, 6.0)	1.0 (91.0, 1.0)	1.0 (91.0, 1.0)
	p	0.25	ND	ND	1.00	1.00
Ang 1–9 (pmol/L)	Pre	2.0 (2.0, 2.0)	2.0 (2.0, 2.0)	2.0 (2.0, 2.0)	2.0 (2.0, 2.0)	2.0 (2.0, 2.0)
	Post	2.0 (2.0, 2.0)	2.0 (2.0, 2.0)	2.0 (2.0, 2.0)	2.0 (2.0, 2.0)	2.0 (2.0, 2.0)
	p	1.00	1.00	1.00	1.00	1.00
Ang 1–7 (pmol/L)	Pre	1.5 (1.5, 1.5)	1.5 (1.5, 1.5)	1.5 (1.5, 1.5)	1.5 (1.5, 1.5)	1.5 (1.5, 1.5)
	Post	5.1 (1.5, 12.5)	1.5 (1.5, 9.2)	23.0 (13.4, 45.7)	7.6 (4.2, 14.5)	1.5 (1.5, 1.5)
	p	<0.001	0.25	0.06	0.06	1.00
Ang 1–5 (pmol/L)	Pre	2.5 (2.5, 7.3)	2.5 (2.5, 3.1)	12.3 (2.5, 17.9)	6.5 (4.6, 15.6)	3.8 (2.5, 6.6)
	Post	14.9 (9.0, 25.4)	13.0 (9.1, 20.3)	21.9 (20.2, 49.5)	28.0 (12.1, 34.3)	2.5 (2.5, 8.8)
	p	<0.001	0.002	0.12	0.03	ND
Ang 3–7 (pmol/L)	Pre	1.5 (1.5, 1.5)	1.5 (1.5, 1.5)	1.5 (1.5, 1.5)	1.5 (1.5, 1.5)	1.5 (1.5, 1.5)
	Post	1.5 (1.5, 1.5)	1.5 (1.5, 1.5)	1.5 (1.5, 3.1)	1.5 (1.5, 1.5)	1.5 (1.5, 1.5)
	p	ND	1.00	ND	1.00	1.00
Ang 2–7 (pmol/L)	Pre	1.0 (1.0, 1.0)	1.0 (1.0, 1.0)	1.0 (1.0, 1.0)	1.0 (1.0, 1.0)	1.0 (1.0, 1.0)
	Post	1.0 (1.0, 1.0)	1.0 (91.0, 1.0)	1.0 (1.0, 5.1)	1.0 (1.0, 1.0)	1.0 (1.0, 1.0)
	p	ND	1.00	ND	1.00	1.00
Ang 2–10 (pmol/L)	Pre	1.0 (1.0, 1.0)	1.0 (1.0, 1.0)	1.0 (1.0, 1.0)	1.0 (1.0, 1.0)	1.0 (1.0, 1.0)
	Post	1.0 (1.0, 1.0)	1.0 (1.0, 2.9)	1.0 (1.0, 3.6)	1.0 (1.0, 1.0)	1.0 (1.0, 1.0)
	p	0.12	0.25	ND	1.00	1.00
Aldosterone (pmol/L)	Pre	83.8 (53.4, 149.5)	122.0 (100.8, 156.2)	83.8 (45.9, 178.3)	66.0 (44.3, 72.5)	48.1 (15.8, 177.7)
	Post	170.6 (112.2, 251.7)	173.7 (106.1, 289.8)	195.0 (125.4, 545.8)	184.1 (112.0, 284.8)	119.9 (73.2, 270.1)
	p	<0.001	0.37	0.12	0.03	0.12
AA2	Pre	8.9 (3.2, 15.0)	15.0 (8.9, 46.3)	11.5 (3.4, 48.0)	4.3 (2.5, 7.6)	1.7 (1.2, 8.1)
	Post	3.2 (1.8, 5.7)	4.3 (1.8, 6.1)	1.4 (1.0, 3.4)	2.7 (2.2, 5.0)	8.4 (2.4, 18.0)
	p	<0.001	0.003	0.06	0.16	0.12
PRA (pmol)	Pre	13.1 (8.5, 24.4)	9.6 (5.4, 16.8)	14.9 (7.7, 18.5)	14.0 (11.8, 28.8)	27.8 (11.6, 43.9)
	Post	67.0 (43.0, 114.2)	53.6 (35.2, 134.9)	113.2 (84.0, 297.0)	58.8 (47.8, 95.7)	33.6 (10.6, 61.1)
	p	<0.001	0.002	0.06	0.03	0.25
ACE** activity (ng/mL)	Pre	3.9 (2.7, 6.8)	2.8 (1.8, 4.1)	4.9 (2.1, 6.4)	3.8 (2.9, 5.3)	8.3 (3.6, 10.9)
	Post	5.0 (3.6, 9.1)	4.7 (3.4, 6.9)	4.1 (2.9, 32.9)	6.9 (4.7, 19.3)	5.2 (2.1, 8.3)
	p	0.07	0.15	0.31	0.16	0.37
ACE2 activity (ng/mL)	Pre	16.6 (13.9, 20.4)	15.3 (14.1, 17.2)	17.5 (14.7, 20.0)	16.2 (10.8, 22.8)	20.1 (15.0, 24.5)
	Post	25.2 (20.2, 33.0)	23.9 (22.1, 26.0)	30.9 (26.4, 45.4)	27.0 (17.1, 42.9)	22.1 (18.9, 27.7)
	p	<0.001	0.002	0.06	0.03	0.25
NEP activity (ng/mL	Pre	2.8 (1.2, 5.9)	3.3 (1.2, 5.8)	1.2 (1.2, 5.6)	1.2 (1.2, 5.1)	6.9 (2.4, 9.8)
	Post	3.7 (1.2, 7.9)	1.2 (1.2, 5.2)	3.9 (2.2, 11.2)	4.2 (1.2, 12.4)	7.3 (3.6, 10.1)
	p	0.49	0.37	0.37	0.25	1.00

Note: A-E, Arabians completing a 50-mile endurance ride ($n=10$); TB-R, Thoroughbreds racing 1 1/16th-miles ($n=5$); QH-R, Quarter horses racing 330–500 yards ($n=6$); and TB-TM, Thoroughbreds completing 1-mile submaximal treadmill exercise ($n=4$). RAAS concentrations measured include angiotensin metabolites, (Ang I, angiotensin I; Ang II, angiotensin II; Ang 1–9, angiotensin 1–9; Ang 2–10, angiotensin 2–10; Ang 1–7, angiotensin 1–7; Ang 1–5, angiotensin 1–5; Ang 2–7, angiotensin 2–7; Ang III, angiotensin III; Ang 3–7, angiotensin 3–7; Ang IV, angiotensin IV), aldosterone, AA2, aldosterone/angiotensin II ratio; PRA, plasma renin activity; ACE(±2) activity, angiotensin converting enzyme activity; and NEP activity, neprilysin activity. *Data are reported as median (first, third quartiles); **In all horses, $n=23$. In Arabian horses, $n=8$; ND = not determined (sample size is too small to perform the analysis or most pre- and post-exercise values are not different). Blue highlighted results are statistically different ($p<0.05$).

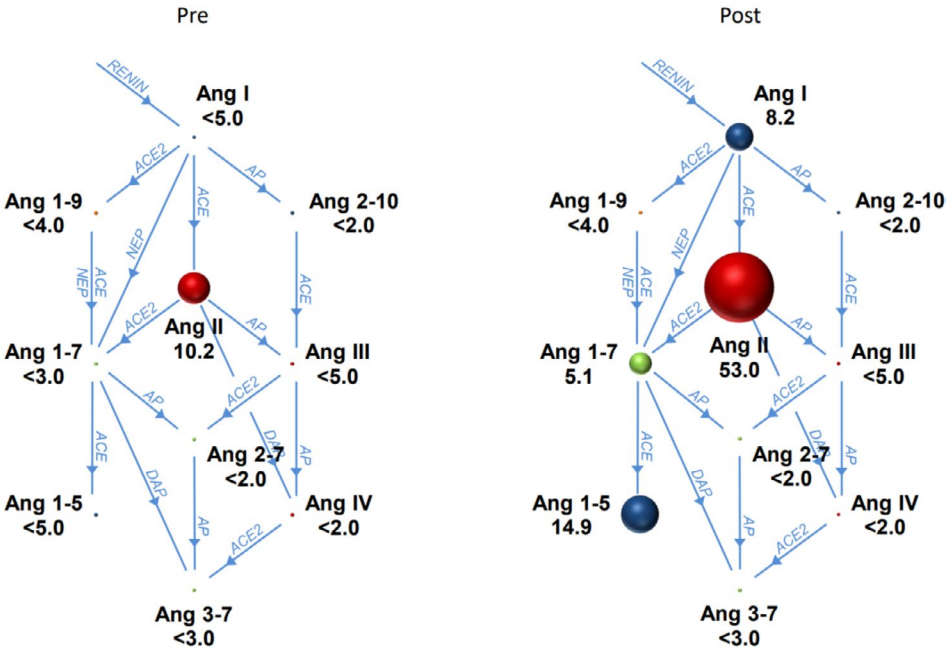

FIGURE 1 | Serum equilibrium concentrations of angiotensin peptides before and after exercise in horses ($n = 25$). Sizes of circles are proportional to the median concentration of the analyte (pmol/L). Ang I, angiotensin I; Ang II, angiotensin II; Ang 1–9, angiotensin 1–9; Ang 2–10, angiotensin 2–10; Ang 1–7, angiotensin 1–7; Ang 1–5, angiotensin 1–5; Ang 2–7, angiotensin 2–7; Ang III, angiotensin III; Ang 3–7, angiotensin 3–7; Ang IV, angiotensin IV.

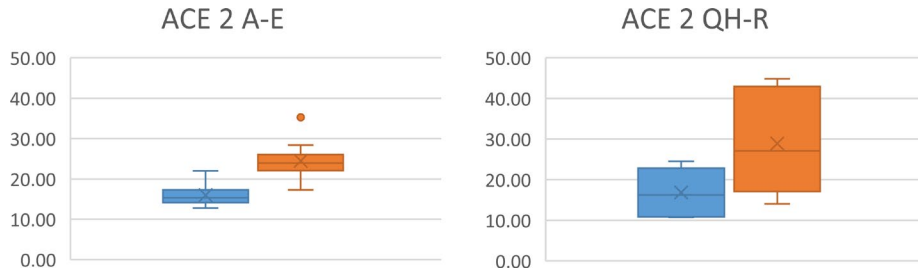

FIGURE 2 | ACE 2 activity (ng/ml) before and after exercise in competing Arabian endurance (A-E; $n = 10$) and racing Quarter horses (Q-R; $n = 6$).

studies comparing different breeds undergoing the same exercise regimen would be required to separate the effect of breed from exercise.

Previous studies found that ACE activity significantly increased in horses exercised to fatigue on a treadmill, whereas endurance exercise did not lead to an increase in ACE activity in field studies [20, 21]. Although ACE activity increased after exercise in our group of horses, the difference did not reach statistical significance. Differences in methodology between studies and sample sizes might have influenced these findings.

To our knowledge, the alternative RAAS metabolites and enzymes have not previously been studied in exercising horses. Similar to humans, exercise in horses induces a significant increase in Ang 1–7 [13, 15]. Our findings support ACE2 as the major formation enzyme for Ang 1–7, without a significant role for NEP in this pathway. The higher ratio of post/pre-exercise Ang 1–7 in TB-R horses compared with A-E and TB-TM groups suggests that near maximal exercise results in more stimulation of ACE2 activity and hence higher Ang 1–7 production. The higher ACE2 activity in the TB-R group compared with the TB-TM group also supports this hypothesis because the TB-TM group was not exercised at a racing pace. Exercise in horses also resulted in a significant increase in Ang 1–5, which is a metabolite of Ang 1–7 mediated by the action of ACE [22]. In humans, exercise type influences Ang 1–7 metabolism. Moderate-intensity aerobic exercise led to more stimulation of the counter-regulatory arm of RAAS compared with high-intensity interval exercise [13]. In that study, ACE2 activity increased in response to high-intensity exercise, similar to our findings in horses in which ACE2 post/pre-exercise ratios were higher in TB-R compared with TB-TM [13]. The beneficial effects of exercise are thought to be partially mediated by the stimulation of the counter-regulatory, alternative RAAS axis and production of the vasodilatory peptide Ang 1–7 [23]. In support of this hypothesis are experimental studies in rodents that support the direct role of Ang 1–7 in the modulation of blood pressure, insulin sensitivity, and other cardioprotective consequences of exercise [24–27].

In humans, disruption of the Ang II/Ang 1–7 balance associated with decreased ACE2 activity may play a pivotal role in the pathophysiology of pulmonary arterial hypertension [28]. Activation of ACE2 and Ang 1–7 production improves pulmonary hemodynamics and ameliorates pulmonary vascular remodeling in rodent models of pulmonary arterial hypertension

TABLE 2 | Comparisons of RAAS concentration ratio values (post/pre-exercise) between A-E, TB-R, QH-R and TB-TM horses*.

RAAS	A-E $n=10$	TB-R $n=5$	QH = R $n=6$	TB-TM $n=4$	p
Ang I	2.9 (1.0, 12.7)[a]	7.4 (2.4, 21.9)[a]	2.8 (1.0, 3.4)[a]	1.5 (1.0, 2.5)[a]	0.26
Ang II	6.8 (4.1, 22.1)[a]	12.5 (8.5, 35.8)[a]	4.7 (2.7, 6.7)[a,b]	1.1 (0.7, 1.2)[b]	<0.001
Ang III	1.0 (1.0, 1.0)[a]	1.0 (1.0, 5.2)[a]	1.0 (1.0, 1.0)[a]	1.0 (1.0, 1.0)[a]	0.27
Ang IV	1.0 (1.0, 1.6)[a]	1.0 (1.0, 6.0)[a]	1.0 (1.0, 1.0)[a]	1.0 (1.0, 1.0)[a]	0.56
Ang 1–9	1.0 (1.0, 1.0)[a]	1.0 (1.0, 1.0)[a]	1.0 (1.0, 1.0)[a]	1.0 (1.0, 1.0)[a]	1.00
Ang 1–7	1.0 (1.0, 6.1)[b]	15.3 (8.9, 30.4)[a]	5.0 (2.8, 9.7)[a,b]	1.0 (1.0, 1.0)[b]	<0.001
Ang 1–5	5.2 (3.3, 7.9)[a]	4.7 (1.4, 8.5)[a,b]	2.3 (1.6, 7.0)[a,b]	1.0 (0.5, 1.8)[b]	0.03
Ang 3–7	1.0 (1.0, 1.0)[a]	1.0 (1.0, 2.1)[a]	1.0 (1.0, 1.0)[a]	1.0 (1.0, 1.0)[a]	0.27
Ang 2–7**	1.0 (1.0, 1.0)[b]	1.0 (1.0, 5.1)[a]	1.0 (1.0, 1.0)[b]	1.0 (1.0, 1.0)[b]	0.03
Ang 2–10	1.0 (1.0, 2.3)[a]	1.0 (1.0, 3.6)[a]	1.0 (1.0, 1.0)[a]	1.0 (1.0, 1.0)[a]	0.39
Aldosterone	1.0 (0.7, 4.1)[a]	2.7 (1.6, 4.3)[a]	3.0 (2.4, 3.9)[a]	2.6 (1.6, 5.3)[a]	0.43
AA2-ratio	0.1 (0.08, 0.3)[b]	0.2 (0.07, 0.3)[b]	0.7 (0.5, 1.1)[a,b]	2.2 (1.3, 8.2)[a]	<0.001
PRA	6.4 (3.2, 11.1)[a,b]	11.4 (7.7, 20.9)[a]	4.1 (2.6, 5.6)[a,b]	1.1 (0.8, 1.3)[b]	<0.001
ACE	1.7 (1.6, 2.4)[a]	3.1 (0.8, 7.8)[a]	2.3 (1.0, 4.7)[a]	0.6 (0.4, 0.9)[a]	0.15
ACE2	1.5 (1.2, 1.9)[a,b]	2.1 (1.6, 2.2)[a]	1.6 (1.3, 1.9)[a,b]	1.1 (0.9, 1.4)[b]	0.03
NEP	0.8 (0.3, 1.0)[a]	1.1 (0.7, 8.9)[a]	2.0 (0.9, 4.8)[a]	1.1 (0.4, 5.7)[a]	0.28

Note: A-E, Arabians completing a 50-mile endurance ride ($n=10$); TB-R, Thoroughbreds racing 1 1/16th-miles ($n=5$); QH-R, Quarter horses racing 330–500 yards ($n=6$); and TB-TM, Thoroughbreds completing 1-mile submaximal treadmill exercise ($n=4$). RAAS concentrations measured include angiotensin metabolites, (Ang I, angiotensin I; Ang II, angiotensin II; Ang 1–9, angiotensin 1–9; Ang 2–10, angiotensin 2–10; Ang 1–7, angiotensin 1–7; Ang 1–5, angiotensin 1–5; Ang 2–7, angiotensin 2–7; Ang III, angiotensin III; Ang 3–7, angiotensin 3–7; Ang IV, angiotensin IV), aldosterone, AA2, aldosterone/angiotensin II ratio; PRA, plasma renin activity; ACE(±2) activity, angiotensin converting enzyme activity; and NEP activity, neprilysin activity. *Data are reported as median (first, third quartiles); [a,b]Within each row, groups with different superscripts and color highlights are statistically significant ($p<0.05$). **In all horses Ang 2–7 ratio values = 1.0, except 2/5 TB-R had ratio values = 7.4 or 2.9; in post hoc analysis group comparisons $p>0.05$.

[24, 29]. In horses, pulmonary hypertension develops physiologically during maximal exercise and also pathologically when associated with left heart disease and chronic pulmonary diseases such as asthma [30–33]. Although the role of angiotensin peptide homeostasis in the regulation of pulmonary hemodynamics in horses is unknown, future studies to elucidate the balance of classical and counter-regulatory RAAS metabolites in health and disease are warranted. Our study findings provide a foundation for understanding the role of the RAAS, both classical (vasoconstrictive, sodium-retentive) and alternative (vasodilatory, natriuretic) in the exercising horse. Future studies of horses with exercise-induced pulmonary disease (such as hemorrhage) might investigate whether the balance between classical and alternative RAAS pathways is disrupted in some horses.

Our study had several limitations. The number of horses in each exercise group was low, which might have decreased the power needed to detect group differences for some RAAS components and some exercise groups. Furthermore, the potential impact of medications on RAAS activation could not be determined because all horses except one in the TB-R and QH-R groups received pre-race furosemide. A recent study in resting horses showed that furosemide caused significant increases in Ang I, Ang II, Ang III, Ang IV, and Ang 1–5 4 h after administration [34]. Other potential impacts of management on RAAS activation, such as time of feeding, could not be controlled in our study and may have impacted the results because previous studies have documented postprandial RAAS activation in horses [35]. In our study, horse breeds were aligned with exercise type. Although doing so is consistent with the equine sport industry, some characteristics might be breed-associated and may influence RAAS response. The group differences between TB-R and TB-TM, however, support the differences being related to exercise type and intensity because both of these groups consisted of Thoroughbred horses.

In conclusion, our study indicates immediate global activation of both the classical and alternative RAAS pathways in exercising horses. The type of exercise influenced the magnitude of activation for some RAAS components. These results provide a basis for the future evaluation of horses with exercise-induced abnormalities.

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Disclosure

Authors declare no off-label use of antimicrobials.

Ethics Statement

Approved by the Institutional Animal Care and Use Committees (IACUC) at Oklahoma State University College of Veterinary Medicine (IACUC-20-56) and the University of Florida College of Veterinary Medicine (201808925). Authors declare human ethics approval was not needed.

Conflicts of Interest

Oliver Domenig is employed by Attoquant Diagnostics. Darcy Adin is a consultant for Ceva Santé Animale and Boehringer Ingelheim. The other authors declare no conflicts of interest.

References

1. J. L. Lavoie and C. D. Sigmund, "Minireview: Overview of the Renin-Angiotensin System—An Endocrine and Paracrine System," *Endocrinology* 144 (2003): 2179–2183.

2. M. K. Ames, C. E. Atkins, and B. Pitt, "The Renin-Angiotensin-Aldosterone System and Its Suppression," *Journal of Veterinary Internal Medicine* 33 (2019): 363–382.

3. C. M. Ferrario, L. Groban, H. Wang, et al., "The Renin-Angiotensin System Biomolecular Cascade: A 2022 Update of Newer Insights and Concepts," *Kidney International. Supplement (2011)* 12 (2022): 36–47.

4. F. Fyhrquist and O. Saijonmaa, "Renin-Angiotensin System Revisited," *Journal of Internal Medicine* 264 (2008): 224–236.

5. J. K. Ho and D. A. Nation, "Cognitive Benefits of Angiotensin IV and Angiotensin-(1–7): A Systematic Review of Experimental Studies," *Neuroscience and Biobehavioral Reviews* 92 (2018): 209–225.

6. O. Domenig, A. Manzel, N. Grobe, et al., "Neprilysin Is a Mediator of Alternative Renin-Angiotensin-System Activation in the Murine and Human Kidney," *Scientific Reports* 6 (2016): 33678.

7. W. R. Welches, R. A. Santos, M. C. Chappell, et al., "Evidence That Prolyl Endopeptidase Participates in the Processing of Brain Angiotensin," *Journal of Hypertension* 9 (1991): 631–638.

8. W. R. Welches, K. B. Brosnihan, and C. M. Ferrario, "A Comparison of the Properties and Enzymatic Activities of Three Angiotensin Processing Enzymes: Angiotensin Converting Enzyme, Prolyl Endopeptidase and Neutral Endopeptidase 24.11," *Life Sciences* 52 (1993): 1461–1480.

9. R. Basu, M. Poglitsch, H. Yogasundaram, et al., "Roles of Angiotensin Peptides and Recombinant Human ACE2 in Heart Failure," *Journal of the American College of Cardiology* 69 (2017): 805–819.

10. D. Adin, C. Atkins, O. Domenig, et al., "Renin-Angiotensin Aldosterone Profile Before and After Angiotensin-Converting Enzyme-Inhibitor Administration in Dogs With Angiotensin-Converting Enzyme Gene Polymorphism," *Journal of Veterinary Internal Medicine* 34 (2020): 600–606.

11. O. J. Ziff, A. Covic, and D. Goldsmith, "Calibrating the Impact of Dual RAAS Blockade on the Heart and the Kidney–Balancing Risks and Benefits," *International Journal of Clinical Practice* 70 (2016): 537–553.

12. M. F. M. Costa, F. A. Ronchi, A. Ivanow, A. K. Carmona, D. Casarini, and R. F. Slocombe, "Association Between Circulating Angiotensin-Converting Enzyme and Exercise-Induced Pulmonary Haemorrhage in Thoroughbred Racehorses," *Research in Veterinary Science* 93 (2012): 993–994.

13. D. M. Magalhaes, A. Nunes-Silva, G. C. Rocha, et al., "Two Protocols of Aerobic Exercise Modulate the Counter-Regulatory Axis of the Renin-Angiotensin System," *Heliyon* 6 (2020): e03208.

14. J. F. Gregorio, G. S. Magalhaes, M. G. Rodrigues-Machado, et al., "Angiotensin-(1-7)/Mas Receptor Modulates Anti-Inflammatory Effects of Exercise Training in a Model of Chronic Allergic Lung Inflammation," *Life Sciences* 282 (2021): 119792.

15. R. de Alcantara Santos, V. Guzzoni, K. A. S. Silva, et al., "Resistance Exercise Shifts the Balance of Renin-Angiotensin System Toward ACE2/Ang 1-7 Axis and Reduces Inflammation in the Kidney of Diabetic Rats," *Life Sciences* 287 (2021): 120058.

16. A. Munoz, C. Riber, P. Trigo, et al., "Dehydration, Electrolyte Imbalances and Renin-Angiotensin-Aldosterone-Vasopressin Axis in Successful and Unsuccessful Endurance Horses," *Equine Veterinary Journal. Supplement* 42 (2010): 83–90.

17. K. H. McKeever, K. W. Hinchcliff, L. M. Schmall, et al., "Plasma Renin Activity and Aldosterone and Vasopressin Concentrations During Incremental Treadmill Exercise in Horses," *American Journal of Veterinary Research* 53 (1992): 1290–1293.

18. M. Masri, J. F. Freestone, K. J. Wolfsheimer, et al., "Alterations in Plasma Volume, Plasma Constituents, Renin Activity and Aldosterone Induced by Maximal Exercise in the Horse," *Equine Veterinary Journal. Supplement* 22 (1990): 72–77.

19. J. L. Ward, E. Guillot, O. Domenig, W. A. Ware, L. Yuan, and J. P. Mochel, "Circulating Renin-Angiotensin-Aldosterone System Activity in Cats With Systemic Hypertension or Cardiomyopathy," *Journal of Veterinary Internal Medicine* 36 (2022): 897–909.

20. M. F. De Mello Costa, G. A. Anderson, H. M. Davies, et al., "Effects of Acute Exercise on Angiotensin I-Converting Enzyme (ACE) Activity in Horses," *Equine Veterinary Journal* 44 (2012): 487–489.

21. M. F. De Mello Costa, G. A. Anderson, H. M. Davies, et al., "Circulating Angiotensin Converting Enzyme in Endurance Horses: Effect of Exercise on Blood Levels and Its Value in Predicting Performance," *Equine Veterinary Journal. Supplement* 42 (2010): 152–154.

22. M. Haschke, M. Schuster, M. Poglitsch, et al., "Pharmacokinetics and Pharmacodynamics of Recombinant Human Angiotensin-Converting Enzyme 2 in Healthy Human Subjects," *Clinical Pharmacokinetics* 52 (2013): 783–792.

23. A. Nunes-Silva, G. C. Rocha, D. M. Magalhaes, L. N. Vaz, M. H. Salviano de Faria, and A. C. Simoes E Silva, "Physical Exercise and ACE2-Angiotensin-(1-7)-Mas Receptor Axis of the Renin Angiotensin System," *Protein and Peptide Letters* 24 (2017): 809–816.

24. M. Bertagnolli, K. R. Casali, F. B. De Sousa, et al., "An Orally Active Angiotensin-(1-7) Inclusion Compound and Exercise Training Produce Similar Cardiovascular Effects in Spontaneously Hypertensive Rats," *Peptides* 51 (2014): 65–73.

25. O. Echeverria-Rodriguez, I. A. Gallardo-Ortiz, L. Del Valle-Mondragon, et al., "Angiotensin-(1-7) Participates in Enhanced Skeletal Muscle Insulin Sensitivity After a Bout of Exercise," *Journal of the Endocrine Society* 4 (2020): bvaa007.

26. A. Shah, Y. B. Oh, S. H. Lee, J. M. Lim, and S. H. Kim, "Angiotensin-(1-7) Attenuates Hypertension in Exercise-Trained Renal Hypertensive Rats," *American Journal of Physiology. Heart and Circulatory Physiology* 302 (2012): H2372–H2380.

27. I. A. Gallardo-Ortiz, R. Villalobos-Molina, and O. Echeverria-Rodriguez, "Potential Role of Angiotensin-(1-7) in the Improvement of Vascular Insulin Sensitivity After a Bout of Exercise," *Experimental Physiology* 105 (2020): 600–605.

28. J. Sandoval, L. Del Valle-Mondragon, F. Masso, et al., "Angiotensin Converting Enzyme 2 and Angiotensin (1-7) Axis in Pulmonary Arterial Hypertension," *European Respiratory Journal* 56 (2020): 1902416.

29. Y. X. Wang, M. L. Liu, B. Zhang, E. Q. Fu, and Z. C. Li, "Fasudil Alleviated Hypoxia-Induced Pulmonary Hypertension by Stabilizing the Expression of Angiotensin-(1-7) in Rats," *European Review for Medical and Pharmacological Sciences* 20 (2016): 3304–3312.

30. B. K. Erickson, H. H. Erickson, and J. R. Coffman, "Pulmonary Artery, Aortic and Oesophageal Pressure Changes During High Intensity Treadmill Exercise in the Horse: A Possible Relation to Exercise-Induced

Pulmonary Haemorrhage," *Equine Veterinary Journal. Supplement* 22 (1990): 47–52.

31. V. B. Reef, F. T. Bain, and P. A. Spencer, "Severe Mitral Regurgitation in Horses: Clinical, Echocardiographic and Pathological Findings," *Equine Veterinary Journal* 30 (1998): 18–27.

32. J. D. Bonagura, "Overview of Equine Cardiac Disease," *Veterinary Clinics of North America. Equine Practice* 35 (2019): 1–22.

33. A. Decloedt, H. Borowicz, M. Slowikowska, K. Chiers, G. van Loon, and A. Niedzwiedz, "Right Ventricular Function During Acute Exacerbation of Severe Equine Asthma," *Equine Veterinary Journal* 49 (2017): 603–608.

34. M. L. Lehman, O. Domenig, M. K. Ames, and J. M. Morgan, "Effect of Furosemide on Comprehensive Renin-Angiotensin-Aldosterone System Activity of Thoroughbred Horses," *Journal of Veterinary Internal Medicine* 38 (2024): 3272–3280.

35. L. L. Clarke, V. K. Ganjam, B. Fichtenbaum, D. Hatfield, and H. E. Garner, "Effect of Feeding on Renin-Angiotensin-Aldosterone System of the Horse," *American Journal of Physiology* 254 (1988): R524–R530.