Effects of monoamines formed in the cecum of horses on equine digital blood vessels and platelets

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Objective—To determine in vitro vasoactive potency of monoamines formed in the cecum and found in the systemic circulation of horses.

Sample Population—Segments of digital blood vessels obtained from 6 healthy mixed-breed horses and ponies euthanatized at an abattoir and platelets isolated from 4 healthy ponies.

Procedure—Paired rings of digital artery and vein from the same horse were examined, and isometric tension was recorded. Concentration-response curves for tryptamine (TRP), tyramine (TYR), phenylethylamine (PEA), isoamylamine (IAA), and isobutylamine (IBA) were obtained. Vasoconstrictor mechanisms were investigated for TRP and TYR by the use of antagonists. Washed platelets loaded with [^{3}H]-5-hydroxytryptamine (5-HT) were incubated with monoamines; the amount of radioactivity displaced after 30 minutes was estimated.

Results—TRP, TYR, and PEA were potent constrictors of arteries and veins, with TRP and TYR being more potent in veins than arteries. Constrictions induced by TYR were inhibited by benextramine (α-antagonist) and nisoxetine (neuronal-uptake blocker), whereas TRP responses were inhibited by ketanserin (5-HT receptor antagonist). All 5 amines displaced 5-HT from platelets with the order of potency being TYR > TRP > PEA > IAA > IBA.

Conclusions and Clinical Relevance—Amines from the equine cecum cause digital vasoconstriction. The most potent (TRP and TYR) cause selective venoconstriction. Tyrosine activates predominantly α-adrenoceptors through the release of neuronal norepinephrine, whereas TRP activates 5-HT receptors. All amines tested released 5-HT from platelets. Amines formed in the cecum and released into the systemic circulation warrant additional investigation as trigger factors for digital ischemia and subsequent laminitis. (*Am J Vet Res* 2003;64:1124–1131)

Pathophysiologic mechanisms of acute laminitis in horses have been the subject of much research. Analysis of risk factors associated with clinical occurrence of this disease reveals that gastrointestinal disorders are often found in association with laminitis.[1] Clinical signs of laminitis can be reproduced experimentally by feeding horses an excess of starch and allowing the readily fermentable carbohydrate to reach the cecum.[2] Ingestion of lush grass has been recognized as a risk factor for development of naturally occurring disease. Ingestion of fructans (ie, grass storage carbohydrates) has been implicated in the pathophysiologic mechanisms of laminitis. Indeed, the clinical syndrome can be reproduced experimentally in horses by feeding fructans.[3]

The pathogenesis by which events within the gastrointestinal tract lead to pain and structural instability of the hoof capsule remains a topic of debate and investigation. Currently, 2 main theories have been proposed. First, the hemodynamic theory suggests that events within the intestine trigger changes within the digital circulation that result in ischemia of the sensitive dermal lamellae. On reperfusion of lamellar structures, pain and tissue damage result, leading to the syndrome recognized clinically.[4] A second theory has been proposed to suggest that the primary event in laminitis results from activation of matrix metalloproteinases within the digit, which destroy the bond between the dermal and epidermal lamellae.[5] It has been proposed[6] that an exotoxin derived from *Streptococcus bovis*, a bacterium that proliferates in the cecum when horses are fed an overload of carbohydrate, is responsible for activation of the matrix metalloproteinases within the digit.

When cecal bacteria are provided a substrate of fermentable carbohydrate, gram-positive organisms proliferate at the expense of gram-negative organisms, and they generate lactic acid.[7] For these acidic conditions, activity of bacterial decarboxylase enzymes is upregulated.[8] Decarboxylation of amino acids within the cecum gives rise to the equivalent amine. In another study[9] conducted by our laboratory group, we documented that amines are generated naturally in the equine cecum such that they are found in cecal fluid at micromolar concentrations. In addition, in an in vitro model of carbohydrate overload, the rate of production of some amines increases by 2- to 3-fold over a 24-hour period.[9] We have also documented that these amines can be detected in the systemic circulation of clinically normal ponies.[10]

Many of the amines produced, particularly those arising from aromatic amino acids, have vasoactive effects in other species.[11] The mechanism of action of these dietary amines is believed to be through direct activation of vascular smooth muscle receptors or indirectly through displacement of norepinephrine from sympathetic nerve endings or serotonin from platelets. The objective of the study reported here was to deter-

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mine the vasoconstrictor potency of monoamines found in the equine cecum and to relate their vasoconstrictor effects to the concentration of amines measured in the systemic circulation of horses.

Materials and Methods

Isometric tension recording from rings of equine digital arteries and veins—Digital arteries and veins were isolated from 6 adult mixed-breed horses and ponies euthanatized at an abattoir. Material was collected from equids that did not have gross pathologic changes of their limbs or feet. The hind limb of each equid was removed immediately after the animal was euthanatized. A catheter was inserted in the digital artery at the level of the metatarsophalangeal joint, and 120 mL of ice-cold Krebs-Henseleit solution (118mM NaCl, 4.57mM KCl, 1.27mM $CaCl_2$, 1.19mM KH_2PO_4, 1.19mM $MgSO_4$, 25mM $NaHCO_3$, and 5.55mM glucose) was infused into the digital circulation. Tissues containing digital arteries and veins were harvested and transported to the laboratory in ice-cold Krebs solution.

Vessel rings (3 to 4 mm in length) were dissected and prepared for isometric tension recording as described elsewhere.[12] Briefly, vessel rings were placed in organ baths in Krebs solution maintained at 30°C and aerated with 95% O_2 and 5% CO_2. Rings were mounted between 2 parallel stainless-steel wires, 1 of which was attached to an isometric force tranducer.[a] The signal from the force transducer was fed via a bridge amplifier[b] to a dual-channel pen recorder.[c] A resting tension of 2 g was applied to the digital veins, and 3 g was applied to the digital arteries. Preliminary experiments documented that these resting tensions were optimal for vasoconstrictor responses. An equilibration period of 60 minutes was allowed before the addition of amines. Up to 6 vessel rings were examined in any given experiment.

Vasoconstrictor potency of aromatic and aliphatic amines—Rings of digital vein and artery from the same animal were examined in any given experiment. Initially, the response of each vessel to application of Krebs solution in which the sodium component had been exchanged for potassium on an equimolar basis (**depolarizing Krebs solution [DKS]**) was measured. Baseline resting tension was reestablished by washing the vessels several times with Krebs solution. Cumulative concentration-response curves were constructed after addition of tryptamine,[d] tyramine,[e] phenylethylamine,[f] isoamylamine,[g] or isobutylamine.[h] Concentrations of amines used ranged from 1×10^{-10} to 3×10^{-4}M or until a definite maximum response was obtained. Only 1 amine was used on each vessel segment. Vessel segments from 6 equids were tested for their vasoconstrictor response to each of the amines. Vasoconstrictor responses to each amine were expressed as a percentage of the DKS response.

Mechanisms for the vasoconstrictor response to tryptamine and tyramine—Additional experiments were performed to establish vasoconstrictor mechanisms for tyramine and tryptamine. To determine whether the actions of tyramine were dependent on neuronal uptake, cumulative concentration-response curves were obtained in vessel segments bathed in Krebs solution containing 10^{-6}M nisoxetine,[i] a competitive inhibitor of neuronal uptake.[13] Vessel rings were incubated with nisoxetine for 30 minutes prior to the addition of tyramine. Control responses were obtained in a second adjacent segment collected from the same animal; these responses were obtained without incubation in nisoxetine. To establish the involvement of α-adrenoceptors in the response to tyramine, vessel segments were treated with an irreversible α-adrenoceptor antagonist (0.1mM benextramine[14,j]) for 30 minutes. Benextramine was then removed by washing the tissues repeatedly with drug-free Krebs solution. Nisoxetine (10^{-6}M) was then added to the bathing solution 30 minutes prior to construction of the cumulative concentration-response curve to tyramine.

The mechanism of action for tryptamine was investigated in a similar manner. Preliminary experiments revealed that nisoxetine did not affect tryptamine-induced vasoconstriction. Thus, the effect of prior treatment of vessel segments with 10^{-4}M benextramine was examined, as described previously for tyramine. In addition, we examined the effect of inclusion of a **5-hydroxytryptamine (5-HT)$_2$** receptor antagonist (10^{-7}M ketanserin[15,k]) in the Krebs solution on the responses to tryptamine.

Displacement of 5-HT from equine platelets by amines—Platelets were obtained from 4 healthy male adult ponies (2 New Forest, 1 Exmoor, and 1 Shetland pony; age, 7 to 10 years old). Blood samples were collected and centrifuged at $300 \times g$ for 15 minutes to obtain platelet-rich plasma, which was then centrifuged at $2,500 \times g$ for 15 minutes. The platelet pellet was resuspended in PBS solution without calcium or magnesium; platelets were washed and recentrifuged twice before being resuspended at a concentration of 2.2×10^8 platelets/mL in complete **Hank's buffered salt solution (HBSS)** containing 0.1% glucose, 0.125% bovine serum albumin, 4mM sodium bicarbonate, and 10mM HEPES buffer (pH, 7.4). Labeled [^{3}H]5-HT[l] was added to unlabeled 5-HT (100μM in HBSS) to yield activity of 10 μCi/μmol. This solution was added to the platelet suspension in a 1:9 ratio to yield a final 5-HT concentration of 10μM; platelets were incubated for 20 minutes at 37°C. After loading, the platelet suspension was again centrifuged, and excess 5-HT was removed by washing the platelets twice more in PBS solution. Washed platelets were resuspended in complete HBSS at a concentration of 3×10^8 platelets/mL.

Each of the 5 amines was added to duplicate 450-μL aliquots of the platelet suspension to yield concentrations ranging from 10^{-7} to 10^{-2}M. Following incubation for 30 minutes at 37°C, platelets were placed on ice and then centrifuged at $10,000 \times g$ for 10 minutes at 0°C. The supernatant was removed and added to scintillation vials, and 4 mL of scintillant[m] was added. Radioactivity was measured by use of a liquid scintillation counter.[n] Corrections for the efficiency of counting were made by the use of external standardization (allowing number of counts per minute to be converted to number of disintegrations per minute), and a known amount of tracer in scintillant was included in each run of the assay to allow the number of disintegrations per minute per nanomole of [^{3}H]5-HT to be calculated. The amount of [^{3}H]5-HT displaced into the medium by the amines was expressed as number of picograms per 10^6 platelets; negative-control values were obtained by incubating platelets without the amines, and total [^{3}H]5-HT taken up by the platelets was measured by loading platelets with [^{3}H]5-HT and lysing aliquots of washed platelets (freeze-thaw technique) in 1M NaOH.

Determination of platelet activation by amines—To assess whether cecum-derived amines caused activation of platelets, aggregation experiments were performed with washed platelets suspended at a concentration of 2×10^8 platelets/mL in complete HBSS in a dual-channel aggregometer.[o] Effects of 10mM concentrations of the amines were compared with effects of 1nM **platelet activating factor**[p] (**PAF**; positive-control sample)[16] and vehicle alone (HBSS; negative-control sample).

After stirring for 10 minutes at 37°C in the aggregometer, 1mM aspirin[q] was added to the samples, which were then centrifuged at $10,000 \times g$ for 10 minutes. Supernatants were assayed to measure concentrations of thromboxane B_2 by use of a radioimmunoassay method, as described elsewhere.[17]

Statistical analysis—All statistical comparisons were performed by use of a commercial software program.[18,r] Cumulative concentration constrictor-response curves for the amines were fitted by use of nonlinear regression to a sigmoidal dose-response curve with variable slope. The **effective concentration producing 50% of the maximum response** (EC_{50}) and maximal response derived for the response of each vessel segment to each amine were then compared between the artery and vein from a given animal to determine the relative potency (ie, EC_{50}) and efficacy (ie, maximum response) of the amine to cause vasoconstriction of the digital vasculature. The threshold concentration at which each amine caused significant constriction of each vessel was calculated as the point at which the concentration-response curve exceeded the 95% **confidence interval (CI)** of the value computed to be the bottom of the curve. Comparisons between arteries and veins were made by use of a paired Student *t* test. Results were reported as the geometric mean value of the **negative logarithm of the EC_{50} value** (pEC_{50}) and threshold values with 95% CIs and the arithmetic mean ± SD of the maximum response data.

To determine the effects of nisoxetine and nisoxetine plus benextramine or benextramine and benextramine plus ketanserin on the responses to tyramine and tryptamine, respectively, cumulative concentration-response curves were fitted to a single-site equation, as described previously. For tyramine, EC_{50}, maximum response, and Hill slope values were compared with those obtained without nisoxetine (control responses) by use of a paired Student *t* test. For tryptamine incubated with ketanserin, the data were best fit by a 2-site equation (as judged by the sum of squares of the residuals and the occurrence of consecutive points above and below the single-site line). Thus, a 2-site equation was used to define the EC_{50} and maximum response values for both phases of the responses to tryptamine, as described elsewhere.[12]

Data from experiments that examined displacement of 5-HT from platelets were fitted by use of a 1-site nonlinear regression equation,[18] and the threshold concentration at which each amine caused significant displacement of [^{3}H]5-HT from equine platelets was calculated as the point at which the concentration-response curve exceeded the 95% CI for the amount of 5-HT released for the negative-control sample (ie, incubation with vehicle). An EC_{50} value was also calculated, representing the amine concentration that caused displacement of 50% of the total [^{3}H]5-HT taken up by the platelets. Relative effects of the amines on the displacement of [^{3}H]5-HT from platelets were compared by use of a 1-way ANOVA with the Fisher post-hoc test. The effects of 1nM PAF and 10mM of each amine on platelet aggregation and thromboxane B_2 production were compared with the effects of vehicle alone by means of a 1-way ANOVA followed by the Dunnett multiple-comparison post-hoc test.

Results

Effects of amines as vasoconstrictors of equine digital arteries and veins—Of the 5 amines tested as vasoconstrictors of equine digital blood vessels, the aromatic amines tryptamine, tyramine, and phenylethylamine caused concentration-dependent vasoconstriction (**Fig 1**). Isoamylamine and isobutylamine failed to cause an increase in vessel wall tension at the maximum concentration tested (10^{-3}M).

Tryptamine was significantly (P = 0.005) more potent as a vasoconstrictor of digital veins (mean pEC_{50}, 5.97; 95% CI, 5.64 to 6.29) than digital arteries (mean pEC_{50}, 4.42; 95% CI, 3.40 to 5.44), but it had similar efficacy in both vessels (mean ± SD maximum response, 197.2 ± 12.9 and 194.2 ± 12.3% for incubation in DKS for digital veins and arteries, respectively). Mean negative logarithm of the threshold concentration for responses of digital veins to tryptamine was 8.17 (95% CI, 7.48 to 8.87), which was significantly lower than the threshold concentration recorded for digital arteries (mean, 7.56; 95% CI, 7.11 to 8.00). Tyramine also was significantly more potent as a constrictor of digital veins (mean pEC_{50}, 4.23; 95% CI, 4.00 to 4.45) than digital arteries (mean pEC_{50}, 3.92; 95% CI, 3.81 to 4.02), and it also had a similar efficacy in both vessel types (mean maximum response, 110.7 ± 11.0 and 92.4 ± 11.5% for incubation in DKS for digital veins and arteries, respectively). Mean negative logarithm of the threshold concentration of tyramine required to cause vasoconstriction was 5.67 (95% CI, 5.18 to 6.17) for digital veins and 5.83 (95% CI, 5.67 to 5.99) for digital arteries. Phenylethylamine was equipotent as a vasoconstrictor of digital veins and arteries (mean pEC_{50}, 4.05; 95% CI, 3.81 to 4.28 for veins and mean, 3.88; 95% CI, 3.78 to 3.97 for arter-

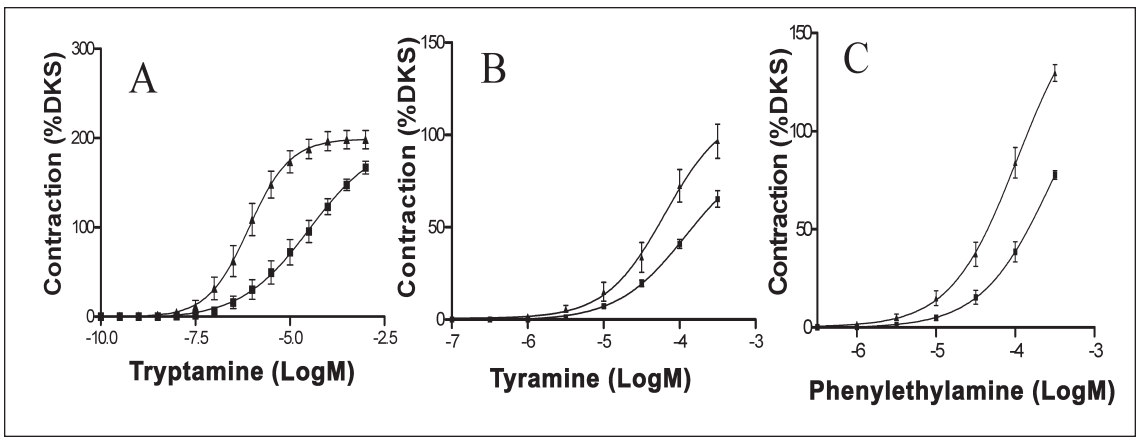

Figure 1—Vasoconstrictor effects of tryptamine (A), tyramine (B), and phenylethylamine (C) on equine digital veins (triangles) and digital arteries (squares). Logarithm of the amine concentration has been plotted against the vasoconstrictor response obtained. Results are expressed as a percentage of the response obtained by incubation in depolarizing Krebs solution (DKS). Each point represents the mean ± SEM obtained for vessels from 6 animals. The line of best fit has been drawn through the points by use of computerized nonlinear regression, and variables for the concentration-response curve were derived. LogM = Logarithmic molar concentration.

ies). Mean threshold concentration for constriction in response to phenylephrine was 5.79 (95% CI, 5.44 to 6.14) for digital veins and 5.71 (95% CI, 5.44 to 5.98) for digital arteries. However, phenylephrine was significantly ($P = 0.005$) more efficacious as a constrictor of veins than arteries (mean maximum response, 166.6 ± 14.9 and 100.4 ± 3.5% for incubation in DKS for digital veins and arteries, respectively).

Effects of antagonists on the responses of digital arteries and veins to tyramine were determined (**Fig 2**). Nisoxetine significantly inhibited responses to tyramine, particularly at the low concentrations tested in the veins, leading to a 2-fold increase in the Hill slope of the concentration-response curve (1.05 ± 0.21 and 2.06 ± 0.46); however, we did not detect a significant change in the EC_{50} or maximum response values. A similar pattern of results was seen in digital arteries, with nisoxetine causing a significant ($P = 0.01$) increase in the Hill slope (0.92 ± 0.08 and 1.28 ± 0.10) and a significant increase in the pEC_{50} (mean, 3.85; 95% CI, 3.49 to 4.20 and mean, 3.32; 95% CI, 3.17 to 3.50); however, it did not significantly affect the maximum response to tyramine. Prior treatment of blood vessels with benextramine substantially reduced the responses obtained to tyramine, leaving only small responses for the 3 highest concentrations of tyramine (ie, 10^{-4}, 3×10^{-4}, and 10^{-3}M). It was not possible to accurately fit concentration-response curves for these data because of the small number of points for each curve.

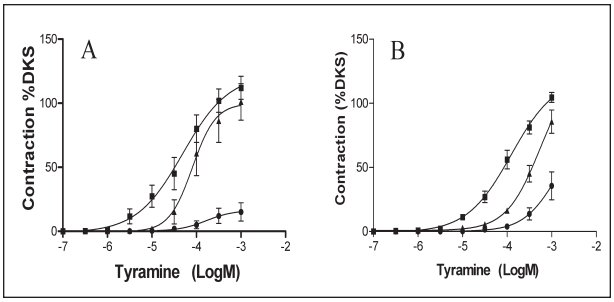

Figure 2—Effects of nisoxitine and benextramine on the vasoconstrictor responses to tyramine for equine digital veins (A) and arteries (B). Responses were obtained for vessel segments incubated without antagonists (control solution; squares), vessel segments incubated with 10^{-6}M nisoxetine (triangles), or vessel segments incubated with 10^{-6}M nisoxetine after segments were treated for 30 minutes with 10^{-4}M benextramine (circles). Each point represents the mean value of responses obtained for vessels from 4 animals. The line of best fit has been drawn through the points by use of computerized nonlinear regression, and variables for the concentration-response curve were derived.

Effects of benextramine and benextramine plus ketanserin on the responses obtained in digital arteries and veins to tryptamine were determined (**Fig 3**). In digital veins, benextramine caused a small and nonsignificant ($P = 0.09$) shift to the right in the concentration-response curve to tryptamine (control pEC_{50}: mean, 6.63; 95% CI, 6.42 to 6.84; benextramine-treated pEC_{50}: mean, 5.86; 95% CI, 5.68 to 6.04) with no change in the maximum response (control, 199.1 ± 19.5% for incubation in DKS; benextramine-treated, 217.1 ± 35.9% for incubation in DKS). A similar pattern was seen in digital arteries, with benextramine not having a significant effect on the pEC_{50} (control: mean, 5.09; 95% CI, 4.50 to 5.68; benextramine-treated: mean, 4.72; 95% CI, 4.27 to 5.17) or the maximum response (control, 177.6 ± 35.1% for incubation in DKS; benextramine-treated, 168.0 ± 61.2% for incubation in DKS). In benextramine-treated vessels, concentration-response curves for tryptamine and ketanserin were biphasic. Mean pEC_{50} for the first phase of the curve was 6.33 (95% CI, 5.47 to 7.19) for the veins and 4.86 (95% CI, 4.64 to 5.07) for the arteries. The first phase had a maximum response of 54.1 ± 12.6% for incubation in DKS for the veins and 53.9 ± 17.8% for incubation in DKS for the arteries. The second phase of the response was clearly to the right of the benextramine-alone curve in veins and arteries with mean

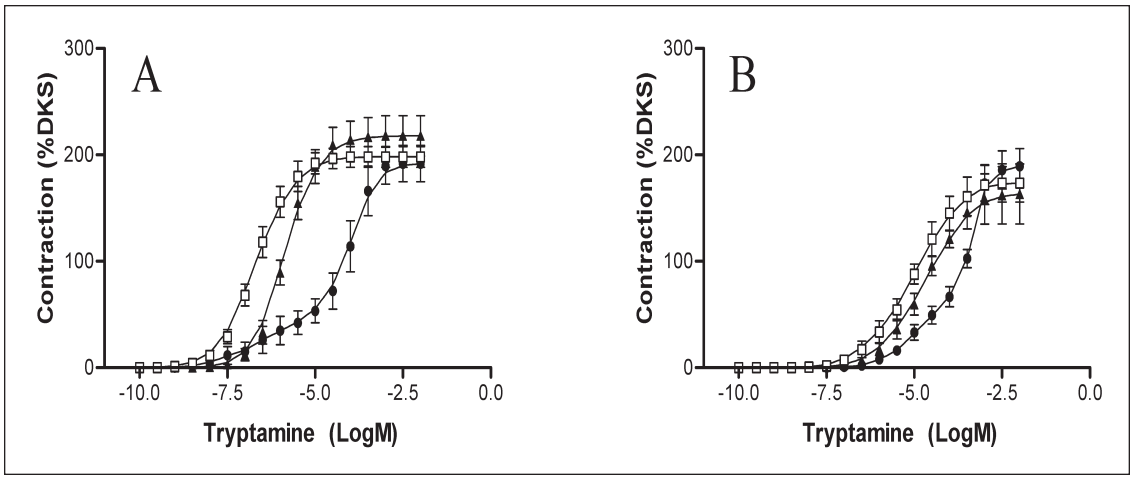

Figure 3—Effects of benextramine and ketanserin on the response to tryptamine for equine digital veins (A) and arteries (B). Responses were obtained for vessel segments incubated without antagonists (control solution; squares), vessel segments treated for 30 minutes with 10^{-4}M benextramine (triangles), or vessel segments incubated with 10^{-7}M ketanserin that had been previously treated with 10^{-4}M benextramine (circles). Each point represents the mean value of responses obtained for vessels obtained from 4 animals. The line of best fit has been drawn through the points by use of a computerized nonlinear regression (single-site equation for control and benextramine-treated tissues; 2-site equation for tissues treated with benextramine plus ketanserin), and variables for the concentration-response curve were derived.

pEC_{50} of 3.93 (95% CI, 3.63 to 4.23) and 3.41 (95% CI, 3.15 to 3.68) for veins and arteries, respectively. Added together, the 2 phases produced the same maximum response as the single-site curve obtained for benextramine-treated tissues.

Displacement of 5-HT from equine platelets by amines—All amines examined caused the displacement of [^{3}H]5-HT from equine platelets (**Fig 4**). Tyramine was the most potent amine, causing significant [^{3}H]5-HT release at a concentration of 0.8µM, and the aromatic amines (ie, tyramine, tryptophan, and phenylethylamine) caused displacement at lower threshold concentrations than did the aliphatic monoamines (ie, isoamylamine and isobutylamine; **Table 1**).

Determination of platelet activation by amines—None of the amines caused significant platelet aggregation at 10mM concentrations, compared with results for the vehicle alone, whereas 1nM PAF caused a marked aggregatory response (29.75 ± 1.30 mV deflection) that differed significantly from the value (1.0 ± 0.25 mV deflection) for the vehicle control sample. Similarly, although PAF caused significant production of thromboxane from the platelets (13.99 ± 1.71 ng/10^9 platelets, compared with 0.74 ± 0.15 ng/10^9 platelets for the vehicle control sample), none of the amines caused significant thromboxane production, compared with results for the vehicle control sample.

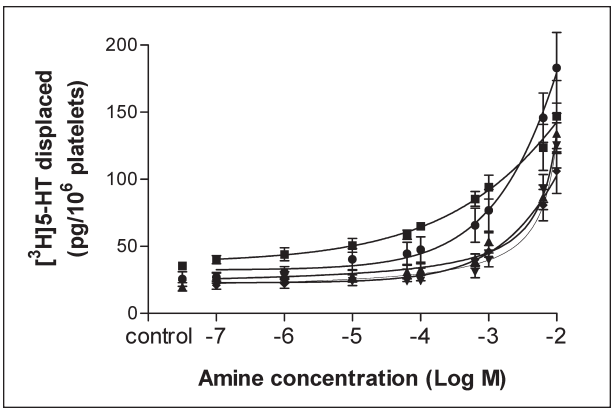

Figure 4—Displacement of [^{3}H]5-hydroxytryptamine (5-HT) from equine platelets by amines found in the cecum. Platelets were loaded with [^{3}H]5-HT and then incubated with tyramine (square), tryptamine (circle), phenylethylamine (diamond), isoamylamine (triangle), or isobutylamine (inverted triangle). Each point represents mean ± SEM of results obtained for platelets from 4 ponies. The line of best fit has been drawn through the points by use of a computerized nonlinear regression, and variables for the concentration-response curve were derived.

Table 1—Geometric mean (95% confidence interval) values* for threshold and effective concentration producing 50% of the maximum response (EC_{50}) for the displacement of [^{3}H]5-hydroxytryptamine (5-HT) from equine platelets by amines produced in the equine cecum

Amine	Threshold†	EC_{50}‡
Tyramine	6.17 (5.42 to 6.94)[a]	2.91 (2.37 to 3.45)
Tryptamine	5.87 (5.47 to 6.27)[a]	2.57 (2.19 to 2.95)
Phenylethylamine	5.31 (4.01 to 6.62)[a]	2.41 (1.93 to 2.88)
Isoamylamine	4.08 (3.59 to 4.57)[b]	2.27 (2.11 to 2.43)
Isobutylamine	3.27 (3.09 to 3.44)[b]	2.37 (2.19 to 2.56)

*Each value represents the geometric mean (95% confidence interval) of the negative logarithm of the molar amine concentration obtained from 4 separate determinations. †Threshold values were calculated from the point at which the concentration-response curve for each amine intercepted the upper 95% confidence interval for the amount of [^{3}H]5-HT displaced in the negative-control sample. ‡The EC_{50} values represent the amine concentration at which 50% of the total amount of [^{3}H]5-HT taken up had been displaced.

[a,b]Values with different superscript letters are significantly ($P < 0.05$) different (1-way ANOVA with the Fisher post-hoc test).

Discussion

Events in the gastrointestinal tract that trigger the pathologic disturbances underlying laminitis have been the subject of much study and debate in veterinary science.[19] A number of hypotheses have been proposed regarding the nature of trigger mediators released from the gastrointestinal tract and the pathophysiologic mechanisms by which they cause laminitis. Supporters of the theory that connective-tissue damage is the primary event in laminitis[5,20] propose that bacterial exotoxins absorbed from the gastrointestinal tract are delivered to the digital circulation and trigger damage to the laminae by activating matrix metalloproteinase enzymes and causing breakdown of the bond between dermal and epidermal lamellae.[6] This theory is supported by the fact that increased matrix metalloproteinase enzymes are activated in laminar tissue collected from acutely laminitic horses.[21] However, ischemia activates matrix metalloproteinase enzymes even prior to reperfusion.[22,23]

Thus, the second major theory of the pathophysiologic mechanisms of laminitis, namely that it represents an ischemia-reperfusion injury, does not exclude matrix metalloproteinase activation as an important component of the disease. Proposed mediators of ischemia that emanate from the gastrointestinal tract include endotoxins released from gram-negative organisms that die in the large intestine as gram-positive organisms (lactobacilli and *S bovis*) proliferate and ferment soluble carbohydrate.[7] However, hemodynamic changes in the digits following infusion of endotoxin do not mimic those that accompany the carbohydrate-overload model for inducing acute laminitis,[4,24] although low doses of endotoxin can significantly reduce digital laminar perfusion[25] and, thus, could contribute to digital ischemia, which is believed by some to precede the onset of clinical laminitis.[26] The exact pattern of hemodynamic changes that accompany in vivo models for inducing acute laminitis in horses has also been the subject of some controversy because of the difficulty in studying laminar perfusion in a noninvasive manner. Results for invasive techniques have suggested that venoconstriction occurs during the prodromal stages of laminitis in animals in which laminitis is induced by use of the carbohydrate-overload model.[27] Thus, it seems logical to suggest that vasoactive mediators involved in triggering digital ischemia that precedes the onset of acute laminitis would act selectively on the digital circulation and, perhaps, have a preferential action on the venous side of that circulation.[4]

The observation that amines, a group of potentially vasoactive compounds formed when bacteria decarboxylate amino acids, are found in the equine cecum at concentrations > 1µM and are detectable in plasma[10] led to the study reported here. If the amines found in the large bowel of horses were potential trigger factors

in acute laminitis, they would need to have vasoconstrictive effects on the digital vasculature at concentrations that are close to those found in plasma. The free concentration of serotonin in plasma is close to the threshold that causes vasoconstriction of the digital vasculature but not of the facial or tail arterial supply.[28] In addition, tryptamine, tyramine, and phenylethylamine are effective inhibitors of uptake of serotonin into platelets[29] and endothelial cells[30] at low micromolar concentrations.

In the study reported here of the amines found in the equine cecum, the primary monoamines were examined for their vascular effects, because they most closely resemble the naturally occurring vascular mediators norepinephrine and serotonin. Of those tested, only the aromatic compounds tryptamine, tyramine, and phenylethylamine had vasoconstrictor actions at submillimolar concentrations. All 3 aromatic amines had some selectivity for the digital veins, although this was most marked for tryptamine, which was 1.55 logarithmic units more potent for the veins, compared with results for the arterial vessels, representing a > 35-fold difference in sensitivity.

The threshold concentration at which vasoconstriction to the aromatic amines was first observed was in the range of 1.5 to 2.5µM for tyramine and phenylethylamine. This is much greater than the measured plasma concentration for these 2 amines in clinically normal ponies and ponies with laminitis, which ranges from 29 to 69nM and 5 to 40nM for phenylethylamine and tyramine, respectively.[10] The threshold concentration of tryptamine at which vasoconstriction was first detected (6.7nM in the veins and 27.6nM in the arteries) was much closer to the recorded plasma concentration of 3 to 20nM in ponies eating spring grass.[10] Together, these findings suggest that relatively small increases in plasma concentrations of tryptamine may result in substantial disturbances in digital hemodynamics. Other observations lend support to this suggestion. Infusion of tryptamine at a rate of 1.6 µg/kg/min into adult Thoroughbreds causes a reduction in hoof-wall surface temperature and decrease in flow in the digital artery, which is significantly different after 15 minutes, without causing any change in systemic arterial blood pressure or heart rate.[s]

We conducted additional experiments to determine the mechanism of the vasoconstrictor action for tyramine and tryptamine. Tyramine is characterized as an indirectly acting sympathomimetic amine that is capable of entering sympathetic nerve endings through a neuronal-uptake carrier mechanism, displacing norepinephrine from neuronal vesicles, and producing vasopressor effects through the release of norepinephrine.[31] Classically, blockade of the neuronal-uptake mechanism will inhibit the action of tyramine. Nisoxetine inhibits the neuronal-uptake process.[13] At the concentration used in the study reported here, it did not cause an effect on the responses of equine digital arteries and veins to exogenously applied norepinephrine (data not shown), but it significantly inhibited responses of both vessel types to tyramine, particularly at the lowest concentrations of tyramine tested, supporting the suggestion that at least part of the response to tyramine was indirect through the release of norepinephrine from sympathetic nerve terminals.

Involvement of α-adrenoceptors in the response to tyramine was confirmed in the study reported here by the effect of the irreversible α_1- and α_2-adrenoceptor antagonist benextramine.[14] The concentration used in our study completely abolished responses of the digital veins to concentrations of norepinephrine up to 10^{-5}M (data not shown), confirming blockade of α_1- and α_2-adrenoceptors, which are maximally stimulated by this concentration of norepinephrine.[32] The small responses to tyramine that remained in benextramine-treated vessels were, therefore, likely to be mediated by receptors other than α-adrenoceptors, but they represent a minor component of the response to tyramine evident only at concentrations of 10^{-4} to 10^{-3}M.

Responses of blood vessels to tryptamine in other species are believed to predominantly involve direct activation of 5-HT receptors with some contribution from activation of α-adrenoceptors.[33,34] In the study reported here, preliminary experiments revealed that nisoxetine failed to inhibit the action of tryptamine, ruling out an indirect action through the release of norepinephrine. Prior treatment of vessels with benextramine resulted in a small shift of the tryptamine concentration-response curve to the right in both veins and arteries, although the EC_{50} value did not change significantly. This lack of a significant change may well have been attributable to the small number of vessels included in this part of the study. Nevertheless, it is possible to conclude from this aspect of the study that the major portion of the response to tryptamine was resistant to inhibition by benextramine and, thus, probably represented direct activation of 5-HT receptors.

Other research conducted by our laboratory group has documented at least 2 types of 5-HT receptors on digital arteries and veins, namely 5-HT_2 receptors and $5\text{-HT}_{1B/D}$ receptors.[12] Incubation with ketanserin, a 5-HT_2-selective receptor antagonist,[15] confirmed that tryptamine activates 2 types of receptors, because there was a ketanserin-resistant and a ketanserin-sensitive component to the response in benextramine-treated tissues, leading to a biphasic concentration-response curve. We did not use antagonists in the study reported here to characterize the nature of the receptor responsible for the ketanserin-resistant component of the response to tryptamine, but it seems likely to be a $5\text{-HT}_{1B/D}$ receptor described in these blood vessels.[12] The proportion of the total response to tryptamine that was mediated by this receptor was approximately 30% in both arteries and veins. However, it is recognized that amplification of the response to stimulation of $5\text{-HT}_{1B/D}$ receptors occurs when there are threshold concentrations of other vasopressor agents, including thromboxane, norepinephrine, and endothelin.[35] Indeed, such synergism has been documented in equine digital arteries and veins by use of subthreshold concentrations of thromboxane A_2-receptor agonists.[t] Corelease of tryptamine and endotoxin from the equine gastrointestinal tract would result in platelet activation and release of thromboxane A_2 from platelets and monocytes,[36] which would act synergistically with tryptamine to cause digital vasoconstriction, amplifying the response to stimulation of $5\text{-HT}_{1B/D}$ receptors.

The remaining experiments conducted in the study reported here examined the interaction between platelets and amines found in the large intestine of horses. Platelets have been implicated in pathophysiologic mechanisms of acute laminitis in horses, with hyperaggregabilty of platelets being evident in the prodromal stages of horses with carbohydrate-induced laminitis.[37] Another study[29] conducted by our laboratory group documented that tryptamine, phenylethylamine, tyramine, and isoamylamine inhibit the uptake of 5-HT into equine platelets. In the study reported here, we examined the ability of amines to displace 5-HT from equine platelets. Displacement was seen with all the amines tested, but only tyramine and tryptamine displaced 5-HT at submicromolar concentrations. However, the concentrations of tyramine and tryptamine required were considerably higher than those we measured in the circulation of ponies eating spring grass.[10] None of the amines tested caused direct activation of platelet aggregation, even when used at concentrations of 10mM.

Aromatic amines found in the cecum and plasma of clinically normal ponies can cause concentration-dependent vasoconstriction of equine digital arteries and veins. Tryptamine was the most potent amine relative to its circulating plasma concentration, such that small increases in circulating tryptamine concentrations would likely cause substantial hemodynamic disturbances in the digits of horses. Tryptamine can act by direct activation of 2 types of 5-HT receptor. When excess amounts of soluble carbohydrate reach the equine cecum, proliferation of lactic acid-producing bacteria leads to a decrease in cecal pH[7] and an increase in intestinal permeability, including the colon.[38] The increased access of a mixture of amines found in the large bowel to the systemic circulation could interact through various mechanisms to produce substantial digital ischemia. On the basis of results of the study reported here, additional investigation of the potential of aromatic monoamines formed in the equine cecum to act as trigger factors for laminitis is warranted.

[a]HSE 30 isometric force transducer, Hugo Sachs Electronics, March-Hugstetten, Germany.
[b]HSE bridge amplifier, Hugo Sachs Electronics, March-Hugstetten, Germany.
[c]Linseis 650 dual-channel pen recorder, Linton Instruments, Diss, Norfolk, UK.
[d]Tryptamine hydrochloride, Sigma Chemical Co, Poole, UK.
[e]Tyramine hydrochloride, Sigma Chemical Co, Poole, UK.
[f]β-phenylethylamine hydrochloride, Sigma Chemical Co, Poole, Dorset, UK.
[g]Isoamylamine, Sigma Chemical Co, Poole, UK.
[h]Isobutylamine, Sigma Chemical Co, Poole, UK.
[i]Nisoxetine hydrochloride, Research Biochemicals Int, Natik, Mass.
[j]Benextramine hydrochloride, Research Biochemicals Int, Natik, Mass.
[k]Ketanserin tartrate, Research Biochemicals Int, Natik, Mass.
[l][^{3}H]5-hydroxytryptamine creatinine sulphate, New England Nuclear, Boston, Mass.
[m]Ecoscint H, National Diagnostics Ltd, Hull, UK.
[n]Packard Tricarb model 4530, Packard Instrument Co, Meriden, Conn.
[o]Payton dual-channel aggregometer module, Payton Associates Ltd, Scarborough, ON, Canada.
[p]Platelet activating factor, Bachem Ltd, St Helens, UK.
[q]Acetylsalicylic acid, Sigma Chemical Co, Poole, UK.
[r]GraphPad Prism, version 3.00 for Windows, GraphPad Software, San Diego, Calif.
[s]Bailey SR, Marr CM, Menzies-Gow N, et al. The effects of vasoactive amines from the equine hindgut on digital blood flow in the normal horse (abstr). *J Vet Intern Med* 2002;16:355.
[t]Bailey SR. *A study of the vascular effects and factors regulating the concentration of 5-hydroxytryptamine in the equine digital circulation*. PhD dissertation, Department of Veterinary Basic Sciences, Royal Veterinary College, University of London, London, UK, 1998.

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