# Roles of thromboxane $A_2$ and 5-hydroxytryptamine in endotoxin-induced digital vasoconstriction in horses

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**Objective**—To evaluate the roles of 5-hydroxytryptamine (5-HT), thromboxane $A_2$ ($TxA_2$), and platelet-activating factor (PAF) in endotoxin-induced digital hypoperfusion in horses.

**Animals**—6 healthy adult Thoroughbreds.

**Procedures**—Horses were treated with IV administration of saline (0.9% NaCl) solution (control treatment) or the $5\text{-}HT_{1B/D}$ selective antagonist, GR55562 (0.3 mg/kg), prior to tryptamine infusion (1.6 µg/kg/min for 30 minutes) to establish an effective GR55562 dose. In a crossover study, horses were treated with IV administration of saline solution (control treatment), aspirin (4 mg/kg, 2 hours or 4 days before lipopolysaccharide [LPS] infusion), GR55562 (0.3 mg/kg), the PAF antagonist WEB2086 (3 mg/kg), or aspirin plus GR55562 prior to LPS infusion (30 ng/kg for 30 minutes). Digital blood flow was measured by use of Doppler ultrasonography. Concomitant measurements of hoof wall and coronary band surface temperatures were made. Serial blood samples were collected and plasma 5-HT and $TxA_2$ concentrations determined.

**Results**—GR55562 abolished tryptamine-induced digital hypoperfusion. Neither WEB2086 nor GR55562 affected LPS-induced alterations in digital perfusion or plasma mediator concentrations. Aspirin given 2 hours before LPS administration abolished the increase in plasma $TxA_2$ concentration and significantly attenuated LPS-induced digital hypoperfusion. Aspirin given 4 days before LPS significantly attenuated the increase in plasma $TxA_2$ concentration and digital hypothermia. Aspirin plus GR55562 had a greater effect on LPS-induced digital hypothermia than aspirin alone.

**Conclusions and Clinical Relevance**—Thromboxane $A_2$ and 5-HT played a role in mediating LPS-induced digital hypoperfusion in horses. Platelet-activating factor appeared unimportant in mediating LPS-induced 5-HT or $TxA_2$ release or digital hypoperfusion. (*Am J Vet Res* 2008;69:199–207)

Endotoxin (ie, LPS) plays a pivotal role in the pathogenesis of a number of diseases in a variety of species and appears to be a potent inducer of tissue hypoxia. For example, endotoxemia is associated with decreases in intestinal villus[1] and cecal blood flow,[2] myocardial ischemia,[3] cerebral hypoperfusion,[4] and impaired renal perfusion and oxygen uptake,[5] whereas skeletal muscle perfusion remains unaffected.[6] In addition, laminitis is a recognized complication of clinical endotoxemia of horses[7] that is thought to be a consequence of altered digital perfusion. The effect of LPS on perfusion may be direct or indirect through the production of vasoactive mediators by LPS-activated cells.

Marked species differences are found in sensitivity to LPS,[8] and humans and horses appear to be exquisitely sensitive. Low-dose LPS infusion in horses[9] is associated with maximum plasma LPS concentrations similar to those reported in carbohydrate overload–induced laminitis during the onset of Obel grade 3 lameness[10] and in horses with acute abdominal disease[11]; clinical signs are consistent with endotoxemia and digital hypoperfusion. Digital hypoperfusion is temporally associated with significant increases in 2 platelet-derived vasoactive mediators, $TxA_2$ and 5-HT.[9] However, the relative importance of these 2 vasoconstrictor mediators in mediating digital hypoperfusion remains unclear. In addition, PAF appears to play a role in the release of these 2 mediators in vitro.[12,13] The aim of the study reported here was to further elucidate the role of $TxA_2$, 5-HT, and PAF in LPS-induced digital hypoperfusion in horses by use of selective inhibitors. Identification

**Abbreviations**

| | |
|---|---|
| LPS | Lipopolysaccharide |
| $TxA_2$ | Thromboxane $A_2$ |
| 5-HT | 5-hydroxytryptamine |
| PAF | Platelet-activating factor |
| CBST | Coronary band surface temperature |
| HWST | Hoof wall surface temperature |
| $AUC_{0–300}$ | Area under the curve between 0 and 300 minutes |

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of the vasoactive mediators responsible for the effects of LPS on digital perfusion might allow the potentially serious effects of digital ischemia to be avoided with the use of selective antagonists in the clinical situation.

Lipopolysaccharide-activated equine leukocytes and platelets release $TxA_2$ and 5-HT in vitro.[13-15] Aspirin prevents equine platelet, leukocyte, and endothelial cell $TxA_2$ synthesis,[16,a] and unlike leukocytes, platelet $TxA_2$ production does not recover for 7 to 10 days following a single dose of aspirin.[16] Thus, aspirin could be used to clarify further the cellular origin as well as the role of $TxA_2$ in LPS-induced digital hypoperfusion in horses in vivo.

Equine digital vessels possess 5-$HT_{1B/D}$ and 5-$HT_2$ receptors that cause vasoconstriction,[17] and synergism between 5-HT and $TxA_2$ in mediating digital vasoconstriction in horses has been determined in vitro.[b] Results of studies[18,19] in other species suggest that this synergism may be the result of $TxA_2$ receptor agonists uncovering the actions of 5-$HT_1$ receptors.The compound 3-[3-(dimethylamino) propyl]-4-hydroxy-N-[4-(4-pyridinyl) phenyl] benzamide dihydrochloride (ie, GR55562) is a potent antagonist for 5-$HT_{1B/D}$ receptors[20]; therefore, GR55562 could be used to investigate the role of 5-HT in LPS-induced digital hypoperfusion in horses. Comparing the effects of simultaneous administration of aspirin and GR55562 with that of aspirin alone would help to establish whether there is synergism between 5-HT acting on 5-$HT_1$ receptors and $TxA_2$.

Platelet-activating factor appears to be involved in the pathogenesis of endotoxemia in horses,[21] and its synthesis may be triggered either directly by LPS or indirectly by cytokines released from LPS-activated cells.[22] Lipopolysaccharide-induced equine platelet aggregation[12] and 5-HT release[13] in vitro are dependent on leukocyte-derived PAF. Thus, if PAF mediates LPS-induced $TxA_2$ and 5-HT release by leukocytes and platelets in vivo, a PAF receptor antagonist should decrease the response to LPS. The compound WEB2086 inhibits PAF-induced equine platelet activation in vitro and ex vivo[23,24] and could therefore be used to investigate the role of PAF and, potentially, 5-HT and $TxA_2$ synergism in LPS-induced digital hypoperfusion in horses.

## Materials and Methods

**Animals**—Experiments were performed under a home office license approved by the Royal Veterinary College Ethics and Welfare Committee. Six healthy adult Thoroughbreds (3 males, 3 females; mean ± SD age, 8.2 ± 2.5 years; weight, 514 to 600 kg) were used.

**Experimental design**—Horses were stabled overnight and acclimatized to a partially temperature controlled environment (mean ± SD ambient temperature, 20.5 ± 2.2°C) for 1 hour before the infusions commenced. Bilateral jugular vein catheters were placed.

In the first experiment, to establish an effective GR55562 dose, tryptamine[c] (1.6 µg/kg/min) was administered for 30 minutes via the left jugular catheter 5 minutes after IV administration of either saline (0.9% NaCl) solution (control treatment) or GR55562[d] (0.3 mg/kg). Tryptamine is an agonist at 5-$HT_1$ and 5-$HT_2$ receptors in equine digital arteries and veins in vitro[25] and causes digital hypoperfusion in vivo.[26]

In the second part of the study, *Escherichia coli* type O55:B5 LPS[c] (30 ng/kg; sterile filtered into 500 mL of saline solution) was administered for 30 minutes via the left jugular catheter. Horses were given one of the following treatment combinations IV: saline solution at 4 days, 2 hours, and 20 minutes before the onset of the LPS infusion and 30 minutes after the onset of the LPS infusion; saline solution at 4 days and 20 minutes before the onset of the LPS infusion and 30 minutes after the onset of LPS infusion and aspirin[c] (4 mg/kg) at 2 hours before the onset of the LPS infusion; aspirin[c] (4 mg/kg) at 4 days before the onset of the LPS infusion and saline solution at 2 hours and at 20 minutes before the onset of the LPS infusion and 30 minutes after the onset of the LPS infusion; WEB2086[e] (3 mg/kg) at 20 minutes before the onset of the LPS infusion and saline solution at 4 days and 2 hours before the onset of the LPS infusion and 30 minutes after the onset of the LPS infusion; saline solution at 4 days, 2 hours, and 20 minutes before the onset of the LPS infusion and GR55562[d] (0.3 mg/kg) at 30 minutes after the onset of the LPS infusion; and aspirin[c] (4 mg/kg) at 2 hours before the onset of the LPS infusion, saline solution at 4 days and 20 minutes before the onset of the LPS infusion, and GR55562[d] (0.3 mg/kg) at 30 minutes after the onset of the LPS infusion.

Doses of aspirin and WEB2086 used are effective in vivo in horses.[23,27] The inhibitory effects of WEB2086 were confirmed by collecting blood (5 mL) into heparin (20 U/mL) from 1 Thoroughbred immediately before and 20 and 90 minutes after IV administration of WEB2086[e] (3 mg/kg). Platelet aggregation in response to PAF (final concentrations, 2nM and 20nM) was measured in platelet-rich plasma as described elsewhere.[23]

Each horse received every pretreatment combination in a randomized order with at least 28 days between experiments. All horses received flunixin meglumine[f] (1.1 mg/kg, IV) at the end of the experiment to inhibit the inflammatory cascade activated by the LPS infusion as a means to prevent further clinical signs associated with LPS infusion.

**Clinical monitoring**—Arterial blood pressure and heart rate were monitored by use of a noninvasive oscillometric blood pressure monitor[g] applied to the middle coccygeal artery. The CBST and HWST were measured every 10 minutes by use of an infrared thermometer[h] focused on the right forefoot (midline), 1 cm above the skin-hoof junction and a third of the way from the coronary band to the toe, respectively, as previously described.[28] Blood flow was measured every 15 minutes for 60 minutes following tryptamine infusion and for 120 minutes and then again at 300 minutes following LPS infusion in the right lateral forelimb digital artery and vein by use of Doppler ultrasonography,[i,j] as previously described.[9] Blood samples (5 mL) were collected into EDTA[c] from horses infused with LPS every 10 minutes for 60 minutes and then every 15 minutes for 4 hours. Leukocyte and platelet counts were subsequently quantified by use of a Coulter counter.[k]

**Plasma 5-HT and TxA2 concentration following LPS infusion**—Blood samples (5 mL) were collected at the same time points into heparinized vacuum tubes containing clomipramine[c] (1 µmol/L) and phenelzine[c] (10 µmol/L), which prevent platelet uptake or metabolism of 5-HT, respectively, and kept on ice. Platelet-poor plasma was obtained from blood samples by centrifugation (300 × *g* for 10 minutes at 4°C and 10,000 × *g* for 10 minutes), and the concentration of 5-HT was measured by use of high-performance liquid chromatography with electrochemical detection, as previously described.[29] Aspirin[c] (1 mmol/L) was added to heparinized blood samples (5 mL) collected at the same time points, and the separated plasma was stored at –80°C. Plasma $TxA_2$ concentrations were subsequently determined indirectly by radioimmunoassay, as previously described.[16]

**Effect of aspirin on ex vivo A23187-induced $TxA_2$ production**—Inhibitory effects of aspirin on platelet and leukocyte $TxA_2$ production were confirmed by collecting blood (5 mL) into EDTA[c] immediately prior to LPS infusion from horses treated with saline solution or aspirin 4 days previously. Platelets and leukocytes were isolated, resuspended in PBS solution[l] to give final concentrations of $1 \times 10^8$ platelets/mL and $1 \times 10^7$ leukocytes/mL, and incubated at 37°C in a shaking water bath for 15 and 60 minutes, respectively, with dimethyl sulfoxide[a] (control treatment) or calcium ionophore A23187[c] (10 µg/mL in dimethyl sulfoxide), as described elsewhere.[15] Cell suspensions were centrifuged (5,000 × *g* for 5 minutes), and the supernatant was removed and stored at –80°C until plasma $TxA_2$ concentrations were determined.

**Statistical analysis**—Values are expressed as mean ± SEM, and statistical analyses were performed by use of computer software.[m] Values from tryptamine infusion were compared among treatment groups by a 2-way ANOVA followed by the Bonferroni post hoc test. For heart rate, blood pressure, and hematologic values from the control (pretreatment with saline solution) LPS infusions, values at each time point were compared with time 0 values by use of a 1-way repeated-measures ANOVA followed by the Dunnett post hoc test. For all variables from LPS infusions, the 5 drug pretreated groups (aspirin 2 hours prior and 4 days prior to LPS, GR55562, WEB2086, and aspirin plus GR55562) were compared with each other and with the control group by use of a 1-way repeated-measures ANOVA followed by the Bonferroni post hoc test. Areas under the curves between 0 and 300 minutes of graphs for digital blood flow, CBST and HWST, and plasma mediator concentration for individual LPS-infused horses were calculated. The effect of pretreatment on $AUC_{0–300}$ was expressed by calculating the percentage difference, compared with the control group, and by comparing the $AUC_{0–300}$ between pretreatment groups by use of a paired Student *t* test. The A23187-induced $TxA_2$ production was compared between saline- and aspirin-treated horses by use of a paired Student *t* test. Values of $P \leq 0.05$ were considered significant.

## Results

**Tryptamine infusions**—Tryptamine infusion, regardless of pretreatment combination, did not result in any significant alterations in heart rate or arterial blood pressure over the study period (data not shown). Following treatment with saline solution, tryptamine infusion caused digital arterial and venous blood flow to decrease by maxima of 39.0 ± 7.5% and 26.0 ± 2.5%, compared with time 0 (start of tryptamine infusion) values, after 45 and 30 minutes, respectively (**Figure 1**). A similar temporal pattern of decreases in CBST and HWST was detected. Treatment with GR55562 (0.3 mg/kg, IV) completely abolished the tryptamine-induced digital hypoperfusion and reductions in CBST and HWST.

**LPS infusions**—Infusion with LPS following treatment with saline solution caused a significant increase in heart rate, compared with time 0 (start of LPS infusion) values, between 45 and 120 minutes, reaching a maximum of 58.2 ± 9.2 beats/min at 75 minutes, but did not cause significant changes in mean, systolic, or diastolic arterial blood pressure (data not shown). Horses had mild signs of abdominal discomfort and tachypnea from 30 to 60 minutes that abated by 90 to 150 minutes, but returned between 210 and 240 minutes and lasted for approximately 30 minutes. Leukocyte and

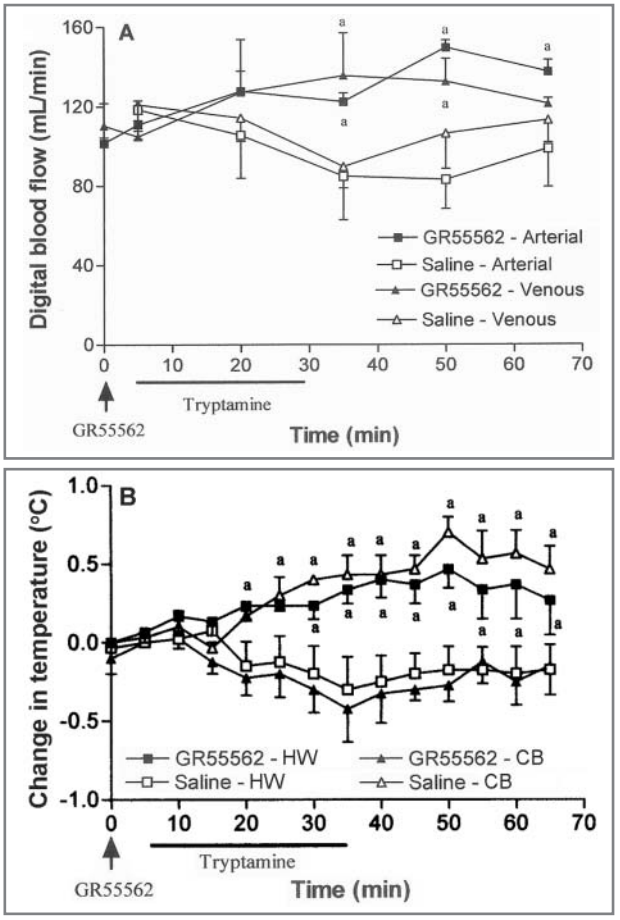


Figure 1—The effect of IV administration of saline (0.9% NaCl) solution and GR55562 (0.3 mg/kg) on tryptamine (480 µg/kg, IV, 30 minutes)-induced alterations in digital arterial and venous blood flow (A) and HWST and CBST (B). Horizontal bar represents time of tryptamine infusion. [a]Significant ($P < 0.05$) difference between treatment groups. Values represent mean ± SEM values from 3 horses. CB = Coronary band. HW = Hoof wall.

platelet counts decreased significantly, compared with time 0 values, between 30 and 120 minutes; the total leukocyte count decreased from 6.4 ± 0.3 × $10^9$ cells/L at time 0 to 2.3 ± 0.3 × $10^9$ cells/L at 120 minutes, and the platelet count decreased from 73.3 ± 11.3 × $10^9$ platelets/L to 54.8 ± 14.9 × $10^9$ platelets/L. Drug pretreatment did not significantly alter the effect of LPS on any of these clinical or hematologic variables (data not shown).

Following treatment with saline solution, LPS infusion caused a significant reduction in digital arterial and venous blood flow, which decreased by maxima of 91 ± 3% and 94 ± 2%, respectively, compared with time 0 (start of LPS infusion) values by 75 minutes; blood flow returned to 95 ± 6% and 88 ± 10% of the original values by the end of the study period (**Figure 2**). Aspirin given 2 hours before LPS significantly attenuated the decrease in blood flow, such that arterial and venous blood flow decreased by maxima of 69 ± 10% and 63 ± 11%, compared with time 0, respectively. Aspirin given 4 days before LPS had no significant effect on the LPS-induced maximum decrease in blood flow. Aspirin given 2 hours before LPS significantly attenuated the overall decrease ($AUC_{0–300}$) in digital blood flow, whereas aspirin given 4 days before LPS had no significant effect (**Table 1**). WEB2086 and GR55562 alone did not affect the LPS-induced maximum (data not shown) or overall alterations ($AUC_{0–300}$) in blood flow at the doses used. In contrast, aspirin plus GR55562 significantly attenuated both of the LPS-induced maximum blood flow decreases, such that arterial and venous blood flow decreased by maxima of 79 ± 5% and 75 ± 5%, compared with time 0 (**Figure 3**) and the overall decrease ($AUC_{0–300}$) in digital blood flow.

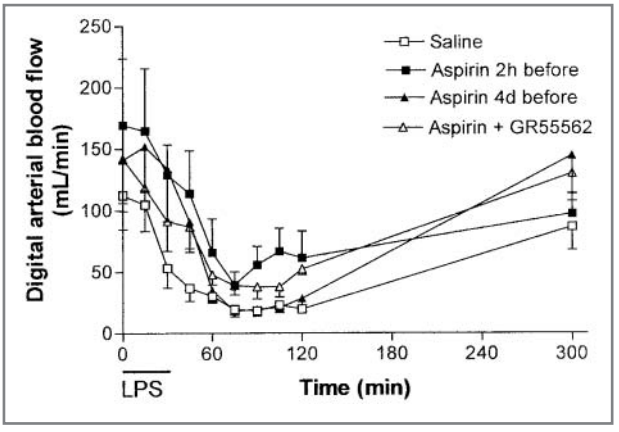


Figure 2—Effect of IV administration of aspirin (4 mg/kg) alone given either 2 hours or 4 days before LPS infusion and aspirin given with GR55562 (0.3 mg/kg) 2 hours before LPS infusion on LPS (30 ng/kg, IV, for 30 minutes)-induced alterations in digital arterial blood flow. Data from horses pretreated with saline solution (control treatment) are shown. Horizontal bar represents time of LPS infusion. Mean ± SEM values from 6 horses.

A similar temporal pattern of reductions in HWST and CBST was detected following administration of LPS to horses pretreated with saline solution (Figure 3). The maximum reduction in HWST and CBST was significantly attenuated to 63 ± 15% and 42 ± 8% and to 57 ± 11% and 41 ± 10% of control LPS infusion–induced changes by aspirin given 2 hours and 4 days before LPS, respectively. In addition, the overall ($AUC_{0–300}$) LPS-induced decreases in HWST and CBST were significantly attenuated (Table 1). No significant difference was found between the attenuation of the LPS-induced changes in HWST and CBST caused by aspirin given 2 hours and aspirin given 4 days before LPS. WEB2086 and GR55562 alone had no significant effect on LPS-induced maximum (data not shown) or overall alterations ($AUC_{0–300}$) in HWST and CBST. Aspirin plus GR55562 significantly attenuated the maximum decreases in HWST and CBST to 19 ± 8% and 33 ± 10%, respectively, of control LPS infusion–induced changes and the overall decrease in HWST and CBST ($AUC_{0–300}$). The attenuation caused by pretreatment with aspirin plus GR55562 was significantly greater than that caused by aspirin given alone 2 hours prior to LPS.

Lipopolysaccharide infusion in horses pretreated with saline solution increased plasma $TxA_2$ and 5-HT concentrations to maxima of 10 and 4 times the baseline value at 55 minutes and 60 minutes, respectively. A secondary increase in plasma $TxA_2$ concentration was observed after 210 minutes, peaking at 240 minutes (**Figure 4**). Plasma 5-HT concentrations returned to baseline values by 180 minutes with no evidence of a secondary increase (data not shown). Aspirin given 2 hours before LPS completely abolished the increase in plasma $TxA_2$. Aspirin given 4 days before LPS caused a significant decrease in $AUC_{0–300}$, compared with the control LPS infusion value (Table 1). Pretreatment with WEB2086 and GR55562 did not decrease the LPS-induced increase in plasma $TxA_2$ concentration significantly. As with aspirin alone, aspirin plus GR55562 completely abolished the effect of LPS on $TxA_2$ production. The LPS-induced increase in plasma 5-HT concentration remained unaffected by any prior treatments.

**PAF receptor antagonist activity of WEB2086—** Pretreatment with WEB2086, at the concentration used, completely inhibited ex vivo PAF (2 or 20nM)-induced

Table 1—Mean ± SEM percentage effect of drug pretreatment on LPS-induced overall change in digital blood flow, HWST and CBST, and plasma mediator concentrations in 6 horses.

| | Blood flow* | | Temperature* | | Plasma concentration* | |
|---|---|---|---|---|---|---|
| **Treatment** | **Arterial** | **Venous** | **HWST** | **CBST** | **$TxA_2$** | **5-HT** |
| Aspirin 2 hours before | 68.8 ± 13.9† | 64.0 ± 13.9† | 63.0 ± 15.0† | 42.0 ± 8.0† | 4.3 ± 1.1† | 109.0 ± 5.8 |
| Aspirin 4 days before | 103.4 ± 8.9 | 108.6 ± 9.2 | 57.0 ± 11.0† | 41.0 ± 10.0† | 57.2 ± 17.4† | 104.0 ± 4.6 |
| WEB2086 | 104.2 ± 5.3 | 102 ± 7.5 | 94.9 ± 8.2 | 100.5 ± 13.5 | 97.9 ± 10.2 | 104.9 ± 4.1 |
| GR55562 | 96.2 ± 5.9 | 99.6 ± 8.1 | 91.7 ± 12.1 | 93.3 ± 8.9 | 103.1 ± 5.2 | 112.9 ± 10.1 |
| Aspirin plus GR55562 | 67.7 ± 7.4† | 62.0 ± 9.5† | 32.9 ± 14.9† | 37.6 ± 13.2† | 3.0 ± 2.0† | 106.2 ± 3.1 |

*Percentage $AUC_{0–300}$ of control LPS infusion values. †Significant ($P < 0.05$) difference, compared with control LPS infusion value.

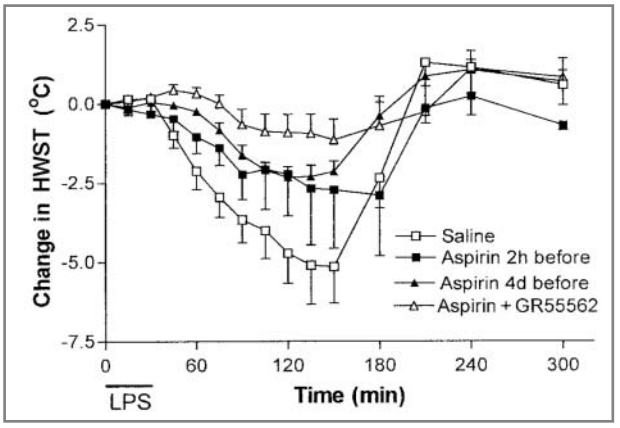


Figure 3—Effect of IV administration of aspirin (4 mg/kg) alone given either 2 hours or 4 days before LPS infusion and aspirin given with GR55562 (0.3 mg/kg) 2 hours before LPS infusion on LPS (30 ng/kg, IV, for 30 minutes)-induced alterations in HWST. Data from horses pretreated with saline solution (control treatment) are shown. Horizontal bar represents time of LPS infusion. Mean ± SEM values from 6 horses.

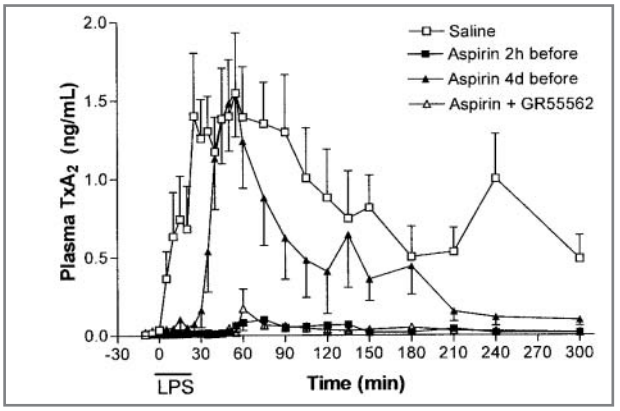


Figure 4—Effect of IV administration of aspirin (4 mg/kg) alone given either 2 hours or 4 days before LPS infusion and aspirin given with GR55562 (0.3 mg/kg) 2 hours before LPS infusion on LPS (30 ng/kg, IV, for 30 minutes)-induced alterations in plasma $TxA_2$ concentration. Data from horses pretreated with saline solution (control treatment) are shown. Horizontal bar represents time of LPS infusion. Mean ± SEM values from 6 horses.

aggregation for at least 90 minutes (37.8 ± 1.6 mV vs 0 ± 0 mV for 20nM PAF at time 0 and time 90 minutes, respectively, n = 1).

**Effect of aspirin on ex vivo A23187-induced $TxA_2$ production—**Use of A23187 caused a 12.0 ± 1.7 fold increase and 12.8 ± 2.1 fold increase in $TxA_2$ production by platelets and leukocytes from horses treated with saline solution, compared with the control solution (0.1% dimethyl sulfoxide), respectively. Aspirin administration 4 days previously significantly decreased ex vivo A23187-induced platelet $TxA_2$ production (4.1 ± 0.9 fold increase), whereas leukocyte $TxA_2$ release was unaltered (15.3 ± 5.8 fold increase).

## Discussion

The aim of the present study was to elucidate further the roles of the vasoactive mediators 5-HT, $TxA_2$, and PAF in LPS-induced digital hypoperfusion in horses by use of selective inhibitors of their formation or action. All 3 mediators are implicated in endotoxin-induced tissue hypoperfusion in a variety of species including horses,[9,30] and synergism between $TxA_2$ and 5-HT in causing digital vasoconstriction in horses is suggested.[b] However, it must be acknowledged that experimental endotoxin infusion and clinical endotoxemia are associated with activation of the systemic inflammatory cascade and the generation of various other vasoactive mediators that may also play a role, as well as hemodynamic changes that were not evaluated in the study reported here.

In vitro, tryptamine is a potent nonselective 5-HT receptor agonist that inhibits equine platelet and endothelial cell 5-HT uptake and releases 5-HT from equine platelets.[24,25,31] When infused into horses at the dosage used in the present study, tryptamine causes an increase in the plasma concentration of 5-HT, which is temporally related to the digital vasoconstriction that ensues.[26] Thus, infusion of tryptamine can be used as a method to evaluate the in vivo effectiveness of potential 5-HT receptor antagonists. GR55562 is a selective $5\text{-}HT_{1B/D}$ receptor antagonist in other species,[20] and equine digital vessels possess $5\text{-}HT_{1B/D}$ and $5\text{-}HT_2$ receptors mediating vasoconstriction.[17] In the present study, the fact that GR55562 completely abolished tryptamine-induced digital hypoperfusion suggested that $5\text{-}HT_1$ receptors mediate the vasoconstriction, thereby negating the requirement to investigate additionally $5\text{-}HT_2$ receptor antagonism. In addition, these findings also suggested that GR55562 at this dose is suitable for use in further evaluating the role of 5-HT acting on $5\text{-}HT_{1B/D}$ receptors in LPS-induced hypoperfusion.

Lipopolysaccharide-activated equine platelets release substantial amounts of $TxA_2$ and 5-HT in vitro,[13,14] and LPS-induced platelet activation is through an indirect mechanism involving calcium, leukocytes, adenine nucleotides, and PAF.[12] Lipopolysaccharide-induced platelet 5-HT release in vitro is inhibited by the PAF receptor antagonist WEB2086 at a concentration of 100nM.[24] Use of WEB2086, when given IV at 3 mg/kg, achieves a concentration in equine plasma of > 100nM for 3 hours[23] and therefore should inhibit PAF-induced platelet 5-HT release in vivo following LPS infusion. In addition, Use of WEB2086 (3 mg/kg, IV) inhibits PAF-induced platelet aggregation ex vivo and inflammatory responses to PAF in vivo.[23,32] This inhibition of ex vivo platelet aggregation was also detected in the present study, confirming that WEB2086 at this dose was suitable for investigation of the role of PAF in LPS-induced vasoactive mediator production in vivo and in digital hypoperfusion in horses.

The 2 techniques used in the present study to quantify digital perfusion were chosen because they are noninvasive techniques that have been used in other studies. Doppler ultrasonography has been used to measure the effect of acepromazine on metatarsal artery blood flow in standing horses,[33] forelimb blood flow in unsedated horses,[34] and arterial digital blood flow during weight bearing and non–weight-bearing conditions.[35] In anesthetized horses, Doppler ultrasonographic measurement of femoral blood flow allows comparison of the hemodynamic effects of inhaled anesthetic agents[36] and determination of the effect of do-

butamine infusion of hind limb perfusion.[37] Peripheral blood flow measurements calculated by use of Doppler ultrasonography correlate well with other invasive techniques including electromagnetic flowmetry,[38] and Doppler ultrasonographic measurements of volumetric flow provide accurate estimates of in vitro blood flow.[39] The repeatability of Doppler ultrasonographic measurement of digital blood flow in horses has also been evaluated.[40] The within and between horse variability was found to be acceptable (coefficients of variation < 11%), and the repeatability was excellent (intraclass correlation coefficients > 0.71) for all variables measured. In addition, power calculations revealed that differences of 0.005 mL/min and 0.01 mL/min in digital arterial and venous blood flow, respectively, would be detected with measurements from 6 horses.[40] Thus, the technique is sufficiently repeatable and sensitive to be able to detect changes in blood flow during different physiologic or pathologic states or following pharmacologic intervention.

Skin-surface temperature is the thermal equilibrium between the external temperature and the exothermic metabolic and mechanical activity and vascular supply of the underlying viable tissues. If measured under conditions of constant moderate ambient temperature and minimal mechanical activity, it is a noninvasive indirect method to assess underlying local perfusion. The technique has been applied to the equine digit in several previous studies[9,26,28,41,42] as an indirect indicator of digital perfusion. Results of these studies indicate that the technique is sensitive enough to detect alterations in digital perfusion following induction of pathologic states[9,28] or pharmacologic intervention.[41,42] In addition, the technique is sufficiently repeatable that the values obtained for HWST and CBST do not change over time following saline solution infusion.[9]

Thus, Doppler ultrasonography was chosen to quantify changes in total blood flow to the equine foot, and HWST and CBST were measured to reflect the underlying microvascular blood flow. Simultaneous use of the 2 techniques should allow effects on total and microvascular blood flow to be differentiated. However, it must be acknowledged that the validity of this assumption has not been investigated and that neither technique distinguishes between blood flow through the arteriovenous anastomoses, sublaminar circulation, or laminar microcirculation.

Following control (pretreatment with saline solution) LPS infusion, digital blood flow decreased, reaching minimum values at 75 minutes and almost returning to pretreatment values by 300 minutes. These decreases in blood flow were similar to those reported in other studies[43,44] and were corroborated by a similar temporal pattern of measurable decreases in surface temperatures, suggesting that total and microvascular blood flow were decreased. In the absence of significant changes in systemic arterial blood pressure, these changes in blood flow may reflect selective digital vascular resistance changes.[9] However, in view of the LPS-induced tachycardia, which is potentially mediated by increased sympathetic tone, the possibility of a generalized systemic response cannot be excluded. Simultaneous measurement of blood flow in other peripheral vessels would be required to determine whether the changes detected in the digital circulation are selective. In agreement with findings of another report,[9] a temporal relationship between LPS-induced increases in plasma 5-HT and $TxA_2$ concentrations and digital hypoperfusion was evident in the present study.

Pretreatment with GR55562 alone did not affect the LPS-induced alterations in digital perfusion or plasma mediator concentrations. The lack of effect of the 5-$HT_{1B/D}$ receptor antagonist on $TxA_2$ production and 5-HT release is unsurprising. The lack of an effect on LPS-induced digital hypoperfusion suggests that 5-HT (acting at 5-$HT_{1B/D}$ receptors) alone is not responsible for the decrease in digital blood flow. The dose of GR55562 used in the present study was sufficient to abolish the digital hypoperfusion resulting from tryptamine infusion. However, infusion of tryptamine caused plasma 5-HT concentrations to approximately double, compared with the baseline value,[26] whereas LPS infusion caused a 4-fold increase in plasma 5-HT concentration. It is possible, therefore, that the dose of GR55562 used in the present study was not sufficient to prevent 5-HT induced vasoconstriction. It is possible that 5-$HT_2$ receptors could be involved in mediating the responses to 5-HT and tryptamine. However, although tryptamine is a nonselective agonist that stimulates 5-$HT_1$ and 5-$HT_2$ receptors, the 5-$HT_2$ receptor antagonist ritanserin does not attenuate tryptamine-induced digital hypoperfusion,[a] suggesting that 5-$HT_1$ receptors are more important in mediating the digital vasoconstriction in vivo.

Aspirin administration significantly attenuated the LPS-induced alterations in plasma $TxA_2$ concentration, but did not affect plasma 5-HT concentration. Although $TxA_2$ is a powerful auto-activator of platelets in other species, it does not appear to have this effect on equine platelets,[14] such that aspirin would not be expected to affect LPS-induced platelet activation and, hence, 5-HT release. Indeed, no effect of aspirin is found on 5-HT release from platelets stimulated with LPS in vitro.[45] Two hours after administration in the study reported here, aspirin caused complete inactivation of platelet and leukocyte cyclooxygenase, as evidenced by complete attenuation of the LPS-induced increases in plasma $TxA_2$ concentration. This finding is in agreement with findings of other studies[16,27,46] evaluating the effect of aspirin on $TxA_2$ production in horses. The abolition of the LPS-induced increases in plasma $TxA_2$ concentration in the present study was accompanied by significant, but not complete, inhibition of LPS-induced digital hypoperfusion and hypothermia. This suggests that leukocyte- and platelet-derived $TxA_2$ are partially responsible for the LPS-induced reduction in total and microvascular blood flow.

Four days after aspirin administration, leukocyte cyclooxygenase activity had recovered, whereas platelet cyclooxygenase remained partially inhibited, as evidenced by the significant reduction in ex vivo platelet, but not leukocyte, responses to the calcium ionophore A23187 and the partial return of the first LPS-induced peak in plasma $TxA_2$ concentration in vivo. Thus, platelets appear to be responsible for the second peak in plasma $TxA_2$ concentration. The LPS-induced total digital hypoperfusion was not changed, but there was a sig-

nificant attenuation of the LPS-induced hypothermia. If HWST and CBST reflect microvascular blood flow, then this suggests that the microvessels, composed of the laminar microcirculation and the sublaminar circulation separated by numerous arteriovenous anastomoses, are more affected by platelet-derived $TxA_2$ than the larger vessels. However, neither noninvasive technique used in the present study to assess perfusion can distinguish between blood flow through the arteriovenous shunts, sublaminar vessels, or laminar vessels. Thus, the attenuation of the digital hypothermia may be a consequence of alteration of blood flow within any of these vessels. If the microvascular blood flow was decreased, total blood flow would also be expected to be decreased. One explanation for this discordance is that the ratio between arteriovenous anastomoses shunt and laminar blood flow is altered, thus attenuating the decrease in HWST and CBST, without an influence on LPS-induced decrease in total blood flow. More direct measures of nutrient versus shunt blood flow, such as microsphere studies, would be necessary to address this issue.

Concomitant administration of aspirin and GR55562 virtually abolished the LPS-induced alterations in plasma $TxA_2$ concentration and unsurprisingly had no effect on plasma 5-HT concentration. The inhibitory effect on LPS-induced digital hypothermia was significantly greater than that detected with aspirin alone when given 2 hours prior to LPS. In addition, LPS-induced digital hypoperfusion was evident only when plasma $TxA_2$ and 5-HT concentrations were increased (ie, 40 to 120 minutes) and not during the second plasma $TxA_2$ concentration peak when the plasma 5-HT concentration was returning to baseline values. Thus, these data might suggest that 5-HT, acting on $5\text{-HT}_1$ receptors, synergizes with $TxA_2$ to mediate LPS-induced alterations in digital perfusion. Synergism between these 2 mediators is found in human platelet aggregation,[47] LPS-induced rat cerebral artery vasoconstriction,[48] vascular smooth muscle cell proliferation,[49] and human[19] and equine[b] digital vasoconstriction in vitro. Results of studies indicate that this synergism is the result of $TxA_2$ receptor agonists uncovering the actions of $5\text{-HT}_1$ receptors[18,19] and may involve activation of multiple signaling pathways, including G-protein–coupled receptor activation of phospholipase C and calcium sensitization.[47] In addition, as evident with aspirin pretreatment alone, a greater effect on surface temperatures was found in the present study, compared with total blood flow, again suggesting that differential sensitivity of the large and microvascular blood vessels exists, with microvascular blood flow being most affected by platelet-derived vasoactive mediators.

Lipopolysaccharide-induced alterations in plasma mediator concentrations were unaffected by WEB2086, suggesting that LPS-induced 5-HT and $TxA_2$ release in vivo is not mediated by PAF, in contrast to other in vitro findings.[12] Alternative explanations include direct platelet activation by LPS, a different leukocyte-derived mediator released in response to LPS activation, or adhesion molecule interactions and subsequent intracellular signaling. Some in vitro evidence exists and suggests that equine platelets are directly activated by LPS,[50] as are human platelets,[51] which express Toll-like receptors.[52] Lipopolysaccharide-stimulated monocyte tissue factor is a potent inducer of platelet activation,[53] although the mechanism remains unclear. At sites of vascular injury, platelets adhere to activated endothelial cells via endothelial P- and E-selectin and the subendothelium and become activated, binding leukocytes via selectins.[54] Upon binding, intracellular signaling is initiated, leading to modulation of several cellular biological properties[55] including, potentially, thromboxane synthase activity. Further studies are required to determine the mechanism of LPS-induced 5-HT and $TxA_2$ release in horses. The lack of effect of WEB2086 on the increase in plasma $TxA_2$ and 5-HT concentrations caused by LPS accounts for the lack of any reduction in digital hypoperfusion. This would suggest that PAF does not play a role in LPS-induced digital hypoperfusion.

Other vasoconstrictor mediators that may be induced by LPS and be responsible for the changes seen in the large vessel blood flow (directly or indirectly) include tumor necrosis factor-$\alpha$; interleukin-1; prostaglandin $F_{2\alpha}$; endothelin-1; and the leukotrienes $C_4$, $D_4$, and $E_4$.[56] Aspirin would be expected to inhibit prostaglandins, and it has been determined that equine plasma endothelin-1 concentrations do not increase following low-dose LPS infusion in horses.[9] However, systemic endothelin-1 concentrations may not accurately reflect local production and activity of endothelin-1 in the microcirculation because > 80% of endothelin-1 released from endothelial cells is secreted abluminally.[57] Further evaluation of the role of the leukotrienes and of endothelial dysfunction in LPS-induced digital hypoperfusion in horses is required. In addition, because of the widespread actions of endotoxin, the possibility that the alterations in blood flow are a consequence of vasoactive mediator generation, neurogenic factors released from sympathetic nerves such as norepinephrine, or hemodynamic pressure–flow factors affecting the systemic circulation must also be considered.

In conclusion, results of the present study on selective inhibitors of mediator formation or action suggest that platelet-derived $TxA_2$ plays a role in LPS-induced digital hypoperfusion and hypothermia in horses and that the sensitivity of the large and microvascular blood vessels to platelet-derived mediators may vary. Inhibition of $5\text{-HT}_1$ receptors alone had no significant effect on the response to LPS but increased the inhibition caused by aspirin, indicating that 5-HT may additionally be involved in mediating vasoconstriction. Platelet-activating factor does not appear to be required for the release of these vasoactive mediators, and further studies are warranted to determine the mechanisms by which LPS affects total digital blood flow.

a. Menzies-Gow NJ. *Endotoxin: role in equine digital hypoperfusion.* PhD thesis, Department of Science, Royal Veterinary College, University of London, 2004.
b. Bailey SR. *A study of the vascular effects and factors regulating the concentration of 5-hydroxytryptamine in the equine digital circulation.* PhD thesis, Department of Science, Royal Veterinary College, University of London, 1998.
c. Sigma, Poole, Dorset, England.
d. Tocris Cookson Ltd, Avonmouth, Bristol, England.
e. Boehringer Ingleheim, Rhein, Germany.

f. Schering-Plough Animal Health, Uxbridge, Middlesex, England.
g. Datascope Accutorr 3/4 series Datasette, Datascope Corp, Paramus, NJ.
h. Model IT-304, Horiba Inc, Kyoto, Japan.
i. Vingmed CFM 800A ultrasound system 5 ultrasound machine, GE Ultrasound, Bedford, England.
j. Echopac image analysis and archiving program, Vingmed Sound, Horten, Norway.
k. Model ZM, Coulter Counter Electronic Inc, Fullerton, Calif.
l. Gibco, Invitrogen Corp, Paisley, Scotland.
m. GraphPad Prism, version 3.00 for Windows, GraphPad Software, San Diego, Calif.

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