Plasma matrix metalloproteinase activity in horses after intravenous infusion of lipopolysaccharide and treatment with matrix metalloproteinase inhibitors

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Objective—To establish an in vivo method for matrix metalloproteinase (MMP)-2 and MMP-9 induction in horses via IV administration of lipopolysaccharide (LPS) and to evaluate the ability of doxycycline, oxytetracycline, flunixin meglumine, and pentoxifylline to inhibit equine MMP-2 and MMP-9 production.

Animals—29 adult horses of various ages and breeds and either sex.

Procedures—In part 1, horses received an IV administration of LPS (n = 5) or saline (0.9% NaCl) solution (5). Venous blood samples were collected before and at specified times for 24 hours after infusion. Plasma was harvested and analyzed for MMP-2 and MMP-9 activities via zymography. In part 2, horses received doxycycline (n = 5), oxytetracycline (5), flunixin meglumine (5), or pentoxifylline (4) before and for up to 12 hours after administration of LPS. Plasma was obtained and analyzed, and results were compared with results from the LPS-infused horses of part 1.

Results—Administration of LPS significantly increased MMP-2 and MMP-9 activities in the venous circulation of horses. All MMP inhibitors significantly decreased LPS-induced increases in MMP activities but to differing degrees. Pentoxifylline and oxytetracycline appeared to be the most effective MMP-2 and MMP-9 inhibitors, whereas doxycycline and flunixin meglumine were more effective at inhibiting MMP-2 activity than MMP-9 activity.

Conclusions and Clinical Relevance—IV administration of LPS to horses caused increased venous plasma activities of MMP-2 and MMP-9. These MMP activities were reduced by pentoxifylline and oxytetracycline, suggesting that further evaluation of these medications for treatment and prevention of MMP-associated diseases in horses is indicated. (Am J Vet Res 2013;74:473–480)
MMPs.17,18 Tetracyclines can downregulate expression of MMP-2 and MMP-9 and may also prevent activation of thezymogen forms.19 Doxycycline, a tetracycline analogue, is used in horses because of its antimicrobial properties, but oxytetracycline is also used for treatment of flexural deformities in foals because of its ability to inhibit MMP-1.23

Both NSAIDs and phosphodiesterase inhibitors decrease MMP-2 and MMP-9 activities by decreasing mRNA expression.20–24 Flunixin meglumine is an NSAID commonly used in horses with pain and inflammation of the gastrointestinal tract. Pentoxifylline, a phosphodiesterase inhibitor, is used in horses with endotoxemia and other systemic inflammatory conditions because of its anti-inflammatory effects.25 To the authors’ knowledge, the effects of flunixin meglumine and pentoxifylline on in vivo MMP inhibition in horses have not been investigated.

The use of effective MMP inhibitors in horses may be beneficial in preventing or lessening diseases associated with MMPs. Experimentally induced endotoxemia in horses has been described,26 and could be a useful method for in vivo MMP induction and subsequent evaluation of MMP inhibitors. The purpose of the study reported here was to determine whether the administration of LPS to conscious horses would increase plasma MMP-2 or MMP-9 activities. If so, experimentally induced endotoxemia could then be used as an in vivo method of MMP induction for evaluation of potential MMP inhibitors (including doxycycline, oxytetracycline, flunixin meglumine, and pentoxifylline) in horses.

**Materials and Methods**

**Horses**—Twenty-nine healthy adult horses (11 mares and 18 geldings) of various breeds (17 Thoroughbreds, 8 Quarter Horses, 3 American Paint Horses, and 1 Arabian) were used in the study. Horses were 3 to 19 years old (median, 7 years) and weighed 379 to 560 kg (median, 491 kg). The horses were free of medical problems related to inflammatory diseases, endotoxemia, or diseases of the digits as determined on the basis of results of a complete physical examination, CBC, lameness examination including hoof testing, and radiographic evaluation of the digits. The study was approved by the Institutional Animal Care and Use Committee of Louisiana State University.

**Experimental design**—The study consisted of 2 parts. In part 1, we evaluated whether the administration of LPS caused production and release of MMPs in the venous circulation of horses. In part 2, we evaluated the effectiveness of several MMP inhibitors on LPS-induced MMP release in the venous circulation of horses.

**Part 1**—Horses were allocated via a randomization procedure (random number table) to a control group (n = 5 horses), which received saline solution, or a treatment group (5), which received LPS. Control horses received 1 L of saline solution IV during a 30-minute period. Treated horses received 1 L of saline solution that contained *Escherichia coli* 055:B5 LPS (35 ng/kg) IV during a 30-minute period. The start of the 30-minute infusion period was designated as time 0.

**Part 2**—Horses were allocated via a randomization procedure (random number table) to receive doxycyclineb (10 mg/kg via nasogastric intubation [n = 5 horses]), oxytetracyclinec (20 mg/kg, IV [5]), flunixin megluminec (1.1 mg/kg, IV [5]), or pentoxifyllinee (8.5 mg/kg diluted in 1 L of saline solution, IV [4]). In addition, horses also received LPS (35 ng/kg) diluted in 1 L of saline solution and administered during a 30-minute infusion period. The MMP inhibitors were administered every 12 hours beginning 12 hours prior to LPS administration and continuing through 12 hours after LPS administration (ie, 3 treatments at –12, 0, and 12 hours). The start of the 30-minute infusion period of LPS was designated as time 0. Results for the horses in these groups were compared with results for the horses that received LPS in part 1.

**Collection of blood samples and monitoring of clinical variables**—For both parts of the study, baseline (time 0) clinical variables were recorded and venous blood samples were collected into heparinized tubes immediately before infusions. After administration of infusions, clinical variables were recorded and venous blood samples were collected at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 16, and 24 hours. Clinical variables monitored were heart rate, respiratory rate, rectal temperature, mucous membrane color, capillary refill time, and behavior. Blood samples were centrifuged for 10 minutes at 3,000 ×g, and the plasma was harvested and stored in 1-mL aliquots at −70°C until analyses for MMP activities and doxycycline concentrations (in doxycycline-treated horses) were performed. Catheters were removed immediately after the blood samples were collected at 24 hours. The horses were observed for an additional 24 hours and then returned to pasture.

**Zymographic analyses of MMP activities**—All zymograms were performed with commercially available
10% gelatin polyacrylamide gels. Venous plasma samples were diluted 1:10 with buffer (23mM Tris, 192mM glycine, and 0.1% SDS; pH, 8.3). The diluted plasma samples as well as pro-MMP-2 and active MMP-2 and pro-MMP-9 and active MMP-9 standards were diluted 1:2 with 2x sample buffer (62.5mM Tris-HCl, 23% glycerol, 4% SDS, and 0.01% bromophenol blue; pH, 6.8). Ten microliters of each sample and 7 µL of each standard were loaded onto each gel. After electrophoresis at 166 V for 1 hour, gels were washed with renaturing buffer (50mM Tris-HCl, 200mM NaCl, 5mM CaCl₂, and 0.02% Brij-35; pH, 7.5) for 30 minutes and then incubated in development buffer (50mM Tris-HCl, 200mM NaCl, 5mM CaCl₂, and 0.02% Brij-35; pH, 7.5) for 16 to 20 hours at 37°C. After incubation, gels were stained with 0.25% Coomassie brilliant blue in a mixture of aqueous 50% methanol and 10% acetic acid (vol/vol) and destained in aqueous 50% methanol and 10% acetic acid (vol/vol). Gelatinolytic activity was detected as transparent bands against a dark-blue background. Relative values in arbitrary units were established for MMP activities by digitally photographing the gels and measuring band intensity and size with densitometry software.

**Results**

**Clinical variables**—Mean values for heart rate, rectal temperature, and respiratory rate for the LPS and saline solution groups in part 1 were summarized (Table 1). Heart rate and rectal temperature did not change significantly from the baseline (time 0) value in horses receiving saline solution; however, respiratory rate increased significantly from the baseline value and remained increased after 0.5 hours in horses receiving saline solution. The administration of LPS significantly increased heart rate from 0.5 through 6 hours and rectal temperature from 1.5 through 6 hours, compared with the administration of saline solution.

Tachycardia, increased rectal temperature, and mild colic were evident in all horses receiving LPS alone or in combination with an MMP inhibitor, which was consistent with a systemic inflammatory response. Horses receiving oxytetracycline or flunixin meglumine had decreased heart rates and rectal temperatures, compared with values for horses receiving only LPS. Horses receiving LPS alone or in combination with an MMP inhibitor had modest, intermittent increases in respiratory rate. However, mean peak values for the MMP in-

Table 1—Mean ± SD heart rate, rectal temperature, and respiratory rate over time for horses receiving an IV infusion of LPS (35 ng/kg) or saline (0.9% NaCl) solution (1 L).
Inhibitors were not significantly different from the value for the LPS group for rectal temperature, heart rate, or respiratory rate (Figure 1). None of the horses in any group developed signs of lameness. Alterations in clinical variables returned to baseline values by 24 hours in all horses.

**Doxycycline assays**—Doxycycline was detected in plasma samples at all time points. A mean ± SD concentration of 0.38 ± 0.17 µg/mL was detected in plasma obtained at baseline (12 hours after the initial doxycycline administration and immediately before administration of doxycycline and LPS). A peak mean concentration of 0.98 ± 0.34 µg/mL was reached 0.5 hours after doxycycline and LPS administration. At 8 hours after doxycycline and LPS administration, the mean doxycycline concentration had decreased to 0.51 ± 0.16 µg/mL.

**Zymographic analyses of MMP activities**—Bands of enzyme activity corresponding to pro–MMP-2 (72 kDa) and pro–MMP-9 (92 kDa) were detected in all venous plasma samples of all horses (Figure 2). The active forms of MMP-2 (66 kDa) and MMP-9 (83 kDa) were not detected.

**Part 1**—Administration of LPS significantly decreased pro–MMP-2 and increased pro–MMP-9 activities in venous plasma over time, compared with baseline values (Figure 3). Both pro–MMP-2 and pro–MMP-9 activities remained unchanged from baseline values in venous plasma samples obtained from horses receiving saline solution. Significant increases in pro–MMP-2 activities for the LPS group, compared with activities for the saline solution group, were delayed and not evident until 16 and 24 hours. Activities of pro–MMP-9 were significantly increased at 0.5, 1, 1.5, 2, 4, 12, 16, and 24 hours for horses receiving LPS, compared with activities at those same times for horses receiving saline solution.

**Part 2**—Mean peak MMP-2 activities for all MMP-inhibitor groups were significantly lower than for the LPS group (Figure 4). Mean peak MMP-9 activities were significantly lower for the oxytetracycline and pentoxifylline groups, compared with activity for the LPS group. Activities of pro–MMP-2 remained unchanged, compared with baseline values, for the doxycycline, oxytetracycline, flunixin meglumine, and pentoxifylline groups; however, pro–MMP-9 activities increased over time, compared with baseline values, despite treatment with an MMP inhibitor in these groups (data not shown). Doxycycline significantly decreased pro–MMP-2 activity in the venous plasma at 0.5, 1, 1.5, 2, 4, 12, 16, and 24 hours and significantly decreased pro–MMP-9 activity at 0.5 and 1.5 hours, compared with activity at those same times in horses receiving saline solution.
Intravenous infusion of LPS significantly increased MMP-2 and MMP-9 activities in the venous circulation of healthy adult horses. As seen in other studies, horses receiving LPS developed clinical signs associated with endotoxemia but did not develop lameness. The administration of doxycycline, oxytetracycline, flunixin meglumine, or pentoxifylline before and during LPS administration resulted in various degrees of significant decreases of MMP-2 and MMP-9 activities in venous plasma.

Gelatin zymography can be used to differentiate between latent and active forms of MMPs and is specific for measuring functional MMP-2 and MMP-9 because they are the only MMPs known to degrade gelatin. The nonproteolytic latent form, but not the active form, of both MMP-2 and MMP-9 were identified in plasma samples. This is similar to results from experimental infusion of LPS in humans and other animals. Reportedly, most of the potential MMP activity in healthy tissue is present in the latent form. Pro–matrix metalloproteinases can undergo allosteric activation without proteolysis of their active domain if they are in contact with the appropriate substrate. Therefore, the zymogen forms may be upregulated and released into the plasma, but they may be awaiting activation by other proteases or seeking the appropriate substrate. In addition, the active MMPs may have already become bound to substrate in tissues and be unavailable for measurement in the plasma.

It is not surprising that LPS administration had more effect on pro–MMP-9 activity than on pro–MMP-2 activity. Endotoxemia incites a severe inflammatory response that initiates numerous mediator cascades, many of which are MMP activators and substrates. Although MMP-9 can be constitutively expressed to some extent, it primarily is induced in response to inflammatory mediators and released by neutrophils. Therefore, MMP-9 seems the mostly likely of the 2 gelatinases to be upregulated by endotoxin exposure. Another study found that endotoxin induces predominantly MMP-9. The constitutive nature of MMP-2 and the lack of response detected in the present study after exposure to LPS lend further evidence to the suggestion that MMP-2 is involved primarily in homeostasis and may possibly even play a protective role. Although there were significant increases in pro–MMP-2 activity at 16 and 24 hours in the LPS group, compared with activities for all other groups, the pro–MMP-2 activities at 16 and 24 hours were not significantly increased,

receiving LPS alone. Administration of pentoxifylline significantly decreased pro–MMP-2 activity in venous plasma at 0, 0.5, 1, 1.5, 2, and 3 hours, compared with activity at those same times in horses receiving only LPS.
compared with the baseline value for the LPS group, and appeared to merely be fluctuations.

All of the MMP inhibitors evaluated in the present study were found to inhibit MMPs in horses. Flunixin meglumine appeared to have the greatest inhibitory effect on plasma MMP-2 activity over time, followed by doxycycline, pentoxifylline, and oxytetracycline; however, pentoxifylline appeared to have the greatest inhibitory effect on mean peak MMP-2 activity, compared with values after LPS administration. In contrast, pentoxifylline was the most effective inhibitor of MMP-9 over time and with regard to mean peak value, with oxytetracycline having only slightly lesser inhibitory effects. Both doxycycline and flunixin meglumine had little inhibitory effect on MMP-9 activity in horses.

The NSAID flunixin meglumine, a nonselective COX inhibitor, had inhibitory effects on both pro–MMP-2 and pro–MMP-9 activities in horses in vivo. These findings differed slightly from results of an in vitro study in which investigators found that neither flunixin meglumine nor phenylbutazone, another NSAID, inhibited equine MMP-2 or MMP-9 obtained from equine cell culture.

The effects of NSAIDs on MMP inhibition have been extensively evaluated. Cyclooxygenase-2 increases activation of MMPs, and NSAIDs (ie, COX inhibitors) decrease MMP-2 and MMP-9 expression. Authors of another study suggest that NSAIDs upregulate mRNA expression of RECK, a membrane-anchored endogenous MMP inhibitor. Increased expression of RECK leads to decreases in MMP-2 activity and suppression of MMP-9 release. Although RECK is inversely related to MMP-2 activation, it is not related to MMP-9 activation. However, tumors with high RECK expression typically have decreased MMP-9 expression. Therefore, flunixin meglumine’s potent inhibitory effects on MMP-2, as opposed to flunixin meglumine’s weak effects on MMP-9, may be attributed to increased expression of RECK and RECK’s apparent affinity for MMP-2 inhibition.

Analysis of results of the present study suggested that pentoxifylline is an effective inhibitor of MMP-9 and MMP-9 activities, compared with activities in horses receiving LPS alone. Similarly, horses receiving oxytetracycline had significantly lower baseline MMP-9 activities. Various cells constitutively produce MMP-2 and, to a small extent, MMP-9. The MMP inhibitors were administered 12 hours before LPS infusion; therefore, it is logical that baseline MMP activities could be decreased because of inhibitory effects already evident at the time of LPS infusion. Inhibition of basal MMP activities suggested that pentoxifylline and oxytetracycline were more potent inhibitors of MMP in horses than were doxycycline and flunixin meglumine. Furthermore, MMP-9 is usually induced by inflammatory mediators and released from neutrophils, as previously stated. The neutrophil-inhibitory effects of pentoxifylline may account for a portion of the decrease in MMP-9 activity detected in the horses of the present study.

Horses receiving pentoxifylline had significantly lower baseline pro–MMP-2 and pro–MMP-9 activities, compared with activities in horses receiving LPS alone. Similarly, horses receiving oxytetracycline had significantly lower baseline pro–MMP-2 and pro–MMP-9 activities. Various cells constitutively produce MMP-2 and, to a small extent, MMP-9. The MMP inhibitors were administered 12 hours before LPS infusion; therefore, it is logical that baseline MMP activities could be decreased because of inhibitory effects already evident at the time of LPS infusion. Inhibition of basal MMP activities suggested that pentoxifylline and oxytetracycline were more potent inhibitors of MMP in horses than were doxycycline and flunixin meglumine. Furthermore, MMP-9 is usually induced by inflammatory mediators and released from neutrophils, as previously stated. The neutrophil-inhibitory effects of pentoxifylline may also have accounted for the decreases in baseline plasma MMP-9 activities.

Although doxycycline is a potent MMP-9 inhibitor in other species and the only drug approved for MMP inhibition in humans, it surprisingly had only minimal effects on LPS-induced MMP-9 activity in the horses of the present study. However, it (along with flunixin meglumine) was an effective inhibitor of MMP-2. This is unusual because other reports indicate that doxycycline predominantly inhibits MMP-9 and in some cases has no inhibitory effect on MMP-2. Inhibition of MMPs by tetracyclines is believed to be via chelation of zinc ions at the binding site in the catalytic domain of MMPs. Another report indicated that tetracyclines regulate MMP gene expression by affecting mRNA stability. Doxycycline may also decrease MMP-9 secretion
through upregulation of its endogenous inhibitor, tissue inhibitor of metalloproteinase-1. 43

Doxycycline reportedly can cause cardiovascular collapse and death when administered IV to horses; therefore, the drug was administered via nasogastric intubation in the present study. Endotoxemia leads to decreased gastric and intestinal motility through the activation of COX and subsequent synthesis and release of prostaglandin E2. 49,50 It is possible that administration of doxycycline via nasogastric intubation in the study reported here may have led to decreased absorption and decreased MMP inhibition. To determine whether there was decreased absorption, plasma doxycycline concentrations were determined. Plasma doxycycline concentrations peaked 0.5 hours after administration via nasogastric intubation, with a mean ± SD of 0.98 ± 0.34 µg/mL. This is consistent with a reported maximum steady-state doxycycline serum concentration of 0.94 µg/mL achieved after oral administration in horses at a dose of 10 mg/kg every 12 hours.11 Therefore, the low degree of MMP inhibition obtained in the present study does not appear to be attributable to decreased intestinal absorption. However, maximum steady-state serum concentrations correspond with the mean inhibitory concentration for certain bacteria. Perhaps serum concentrations of doxycycline required for adequate MMP inhibition in horses are much greater than the mean inhibitory concentration; therefore, the dose used in this in vivo study may not have been sufficient for maximal MMP inhibition. Inhibition of MMPs in humans is evident at dosages less than those used for antimicrobial treatment. 21 Oxytetracycline had a much greater effect on equine MMP inhibition than did doxycycline. Oxytetracycline was both a potent MMP-9 inhibitor and a modest MMP-2 inhibitor. The effects of oxytetracycline on MMP-2 and MMP-9 inhibition have not been studied previously. It appears that oxytetracycline has greater inhibitory effects on MMP-9 than on MMP-2, as has been observed for other tetracyclines.36,47 Tetracyclines are excreted through the biliary duct into the intestine; therefore, there is a reported risk for the development of diarrhea following administration of high doses of tetracyclines because of alterations in intestinal microbes.11 Several studies11-14 have revealed the safety of oral administration of doxycycline at both 10 mg/kg every 12 hours and 20 mg/kg every 24 hours. Oxytetracycline has been used safely in horses at doses ranging from 5 to 40 mg/kg IV.15 In the present study, none of the horses receiving doxycycline at a dose of 10 mg/kg via nasogastric intubation or oxytetracycline at a dose of 20 mg/kg IV every 12 hours developed diarrhea or other adverse effects.

The present study revealed that experimentally induced endotoxemia increases synthesis of pro–MMP-2 and pro–MMP-9 in horses and can be used as a method for evaluating the effectiveness of MMP inhibitors. Specifically, the currently available MMP inhibitors pentoxifylline, oxytetracycline, flunixin meglumine, and doxycycline were all found to be capable of inhibiting equine pro–MMP-2 and pro–MMP-9 to some extent. Flunixin meglumine and doxycycline appeared to be effective inhibitors of MMP-2 and weak inhibitors of MMP-9. However, pentoxifylline and oxytetracycline were both effective inhibitors of MMP-2 and MMP-9 in horses. Pentoxifylline and oxytetracycline caused the greatest overall reduction in MMP activities and warrant further study to evaluate their usefulness as therapeutic or preventive treatments for diseases associated with increased MMP activity in horses.

References


