Effects of equine metabolic syndrome on inflammatory responses of horses to intravenous lipopolysaccharide infusion

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Objective—To test the hypothesis that inflammatory responses to endotoxemia differ between healthy horses and horses with equine metabolic syndrome (EMS).

Animals—6 healthy horses and 6 horses with EMS.

Procedures—Each horse randomly received an IV infusion of lipopolysaccharide (20 ng/kg [in 60 mL of sterile saline (0.9% NaCl) solution]) or saline solution, followed by the other treatment after a 7-day washout period. Baseline data were obtained 30 minutes before each infusion. After infusion, a physical examination was performed hourly for 9 hours and at 15 and 21 hours; a whole blood sample was collected at 30, 60, 90, 120, 180, and 240 minutes for assessment of inflammatory cytokine gene expression. Liver biopsy was performed between 240 and 360 minutes after infusion.

Results—Following lipopolysaccharide infusion in healthy horses and horses with EMS, mean rectal temperature, heart rate, and respiratory rate increased, compared with baseline findings, as did whole blood gene expression of interleukin (IL)-1β, IL-6, IL-8, IL-10, and tumor necrosis factor-α. The magnitude of blood cytokine responses did not differ between groups, but increased expression of IL-6, IL-8, IL-10, and tumor necrosis factor-α persisted for longer periods in EMS-affected horses. Lipopolysaccharide infusion increased liver tissue gene expressions of IL-6 in healthy horses and IL-8 in both healthy and EMS-affected horses, but these gene expressions did not differ between groups.

Conclusions and Clinical Relevance—Results supported the hypothesis that EMS affects horses’ inflammatory responses to endotoxin by prolonging cytokine expression in circulating leukocytes. These findings are relevant to the association between obesity and laminitis in horses with EMS. (Am J Vet Res 2013;74:1010–1019)

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In horses, EMS is characterized by 3 key abnormalities: generalized or regional adiposity, insulin resistance, and laminitis.1 Most affected equids have all 3 problems, but a lean phenotype also exists. Although EMS shares some characteristics with the metabolic syndrome in humans,2 laminitis rather than cardiovascular disease is of primary importance in horses. In humans, it has been established that obesity causes increased secretion of inflammatory cytokines and adipokines from adipose tissue, and this leads to insulin resistance3 and vascular endothelial dysfunction.4 Obese horses, like obese humans, appear to be in a low-grade systemic inflammatory state.5,6 Increased IL-1 and TNF-α expression has been detected in the blood of obese horses,7 and serum TNF-α protein concentrations are higher in obese, laminitis-prone ponies.8 The extent to which equine adipose tissue contributes to systemic inflammation during obesity has only recently been examined, to our knowledge. Research7 to date has demonstrated that cytokine expression is highest in

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nuchal fat, but omental fat is also a site of inflammatory mediator production in horses. 

Anecdotally, it has been suggested that horses with EMS are more prone to development of laminitis when affected by systemic illnesses such as bacterial enterocolitis. In human medicine, obesity is associated with a poorer outcome in patients with sepsis. Evidence suggests that inflammatory responses may be exaggerated in obese individuals, increasing their risk of organ failure. Because endothelial cells are compromised in obese individuals, they appear to be less capable of withstanding damage associated with pathological conditions such as sepsis or ischemia-reperfusion injury. Obesity may render certain vascular beds more vulnerable than others to damage, exacerbating microvascular dysfunction and increasing susceptibility to organ damage at these sites. The inflammatory and vascular events that occur during laminitis share many similarities with sepsis-associated organ failure. It is therefore conceivable that the laminar vasculature may be particularly sensitive to damage from inflammation in obese horses.

Hepatic lipidosis is a manifestation of the metabolic syndrome in humans, and a steatotic liver contributes to systemic inflammation and insulin resistance. Hepatic involvement may also develop in horses with EMS; postmortem hepatic lipid accumulation and increased plasma γ-glutamyl transferase and aspartate aminotransferase activities have been detected in some affected horses. The liver is a major site of toxin clearance from the body, so compromised liver function might contribute to increased inflammation in obese animals. A recent study revealed that hepatic TLR signaling is upregulated in horses with endocrinopathic laminitis, and this further supports a role for hepatic dysfunction in the pathogenesis of this disease.

The purpose of the study reported here was to determine whether the response to an inflammatory stimulus differs between horses with EMS and healthy horses. We hypothesized that EMS would affect the systemic inflammatory response and expression of inflammatory cytokines induced by endotoxin in peripheral blood leukocytes and liver tissues of horses.

Materials and Methods

Animals—Six healthy adult mares and 6 horses with previously diagnosed EMS (3 mares and 3 geldings) from the University of Tennessee teaching and research herd were included in the study. Healthy horses had no history of laminitis, and baseline serum insulin concentrations were < 20 μIU/mL. Horses with EMS had a history of chronic obesity or abnormal regional adiposity and previously documented resting hyperinsulinemia or insulin resistance as confirmed by results of combined glucose-insulin or insulin-modified frequently sampled IV glucose tolerance testing. Abnormal hoof characteristics and radiographic evidence of chronic laminitis were noted for 5 of 6 horses with EMS, and divergent hoof growth rings were detected via physical examination in the sixth horse. Horses with EMS were affected by chronic laminitis; however, these horses were part of an intensively managed research herd and received regular therapeutic farrier care. Therefore, active laminitis was not present in any of the EMS-affected horses at the time of the study. Horses with EMS had been donated to the university 15 to 33 months before the study was performed.

Healthy horses ranged in age from 10 to 18 years (mean age, 12.8 years; median age, 12 years) and weighed 427 to 509 kg (mean weight, 473.5 kg; median weight, 480.5 kg). Breeds included Quarter Horse (n = 3), Quarter Horse cross (1), Appaloosa (1), and Standardbred cross (1). Horses with EMS were 8 to 22 years old (mean age, 15.7 years; median age, 16 years) and weighed 394 to 525 kg (mean weight, 441.8 kg; median weight, 425 kg). Breeds included Paso Fino (n = 2), Morgan (1), Arabian (1), Tennessee Walking Horse (1), and Azteca (1). For all horses, body condition score was assessed by 2 observers on the basis of a scale of 1 to 9 described by Henneke et al, and a mean body condition score was calculated for each horse and for each group. Mean individual body condition scores ranged from 5 to 7.25 (mean group score, 6; median group score, 5.9) for healthy horses and from 4 to 9 (mean group score, 6.3; median group score, 6.4) for horses with EMS.

Horses were paired (1 healthy horse and 1 EMS-affected horse) and housed together in 15 × 55-m dirt paddocks with run-in sheds at the University of Tennessee Cherokee Farm research facility for a minimum of 2 weeks prior to the start of the study. Grass hay and water were provided ad libitum. The study protocol was approved by the University of Tennessee Institutional Animal Care and Use Committee.

Study design—Testing was performed during February through April 2010. A completely randomized design with split plot and crossover in the subplot was used, and horses were evaluated in pairs over 2 consecutive weeks. Three pairs were randomly assigned to receive an IV infusion of LPS in the first week and an IV infusion of sterile saline (0.9% NaCl) solution in the second week. Treatment order was reversed for the remaining 3 pairs of horses. Horses were transported to the veterinary teaching hospital and housed in 3.7 × 3.7-m box stalls at the start of each testing week. Body weight measurements and physical examinations were performed at the time of arrival. Horses were allowed to acclimate to the hospital environment for a minimum of 24 hours prior to testing.

For each horse, a 14-gauge polypropylene IV catheter was aseptically placed in a jugular vein, and a blood sample (20 mL) was collected for a CBC and plasma biochemical analysis at 8:30 AM on day 1 (~27 hours). Baseline physical examination variables were assessed and a blood sample (20 mL) was obtained at 11:00 AM on day 2 (~30 minutes), followed immediately by IV infusion of LPS or saline solution (slow bolus administration over 30 minutes). Conclusion of the IV infusion was designated as 0 minutes. A whole blood sample (2.3 mL) was collected for inflammatory cytokine gene expression analysis at 30, 60, 90, 120, 180, and 240 minutes; at 180 minutes, an additional CBC was performed. A liver biopsy was performed under
standing sedation between 240 and 360 minutes (relative to IV infusion of LPS or saline solution). A physical examination was performed hourly for 9 hours and at 15 and 21 hours following LPS or saline solution administration. Insulin-modified frequently sampled IV glucose tolerance tests were performed at –27 hours, 30 minutes, and 21 hours as part of a concurrent study to assess glucose and insulin dynamics.21 Each horse had access to grass hay and water at all times during testing. The IV catheter was removed after completion of the 21-hour insulin-modified frequently sampled IV glucose tolerance test, and each horse was returned to the research farm. Procedures were repeated the following week, and the alternate treatment (LPS or saline solution) was administered to each horse (total of 2 treatments/horse).

**LPS and saline solution administration**—Commercially available Escherichia coli O55:B5 LPS\(^a\) (20 ng/kg) was diluted in 60 mL of sterile saline solution and administered IV over a 30-minute period. Horses receiving the control treatment were administered 60 mL of sterile saline solution IV over a 30-minute period.

**Measurement of clinicopathologic variables**—Blood samples were collected into appropriate tubes with or without anticoagulant. Samples underwent a CBC, assessment of plasma fibrinogen concentration, and plasma biochemical analysis at the University of Tennessee Clinical Pathology laboratory.

**Liver biopsy**—Each horse was restrained in stocks and sedated. A 15 × 15-cm area overlying the right 12th to 14th intercostal spaces was clipped of hair and prepared with povidone-iodine surgical scrub. The access site was infiltrated SC with 12 mL of 2% mepivacaine hydrochloride, and an ultrasound-guided needle biopsy sy was performed with a 14-gauge spring-action needle biopsy device.\(^b\) Two 1.5-cm biopsy cores were collected from each horse. One core was immediately fixed in neutral-buffered 10% formalin, and the other was snap frozen on dry ice and stored at –80°C until gene expression and histologic examination was performed hourly for 9 hours and at 15 and 21 hours following LPS or saline solution administration. Insulin-modified frequently sampled IV glucose tolerance test, and each horse was returned to the research farm. Procedures were repeated the following week, and the alternate treatment (LPS or saline solution) was administered to each horse (total of 2 treatments/horse).

**Histologic examination of liver biopsy specimens**—Routine H&E staining and periodic acid–Schiff staining were performed on sections of formalin-fixed biopsy specimens. Tissue sections (5-µm thickness) were evaluated with light microscopy by a single veterinary pathologist (RLD) who remained unaware of treatment group allocations. Inflammation, lipid content, and glycogen content were each graded subjectively on a scale of 0 (absent) to 3 (marked).

**Inflammatory cytokine gene expression in whole blood samples**—Blood samples for quantitation of IL-1\(\beta\), IL-6, IL-8, TNF-\(\alpha\), and B2M gene expression were collected into appropriate tubes with or without anticoagulant. Samples underwent a CBC, assessment of plasma fibrinogen concentration, and plasma biochemical analysis at the University of Tennessee Clinical Pathology laboratory.

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**Inflammatory cytokine gene expression in whole blood samples**—Blood samples for quantitation of IL-1\(\beta\), IL-6, IL-8, TNF-\(\alpha\), and B2M gene expression were collected into whole blood RNA collection tubes\(^c\) and allowed to incubate at room temperature (approx 22°C) for 8 hours, then stored at –20°C until analyzed. Total RNA extraction and real-time PCR assays were performed as previously described.\(^{21,22}\) Amplification efficiencies of individual PCR amplifications were verified to be between 80% and 120% with computer software.\(^5\) The comparative cycle threshold (\(\Delta\Delta\)Ct) method was used to determine fold changes in inflammatory cytokine gene expression.\(^23\) Cytokine expression was normalized to the housekeeping gene B-Gus, and each horse’s baseline value was used as the calibrator. Fold changes in gene expression were therefore determined at the level of the individual animal, and baseline gene expression was equal to a 1-fold change for all cytokines.

**Inflammatory cytokine gene expression in liver tissue**—Total RNA was extracted from frozen liver tissue samples with a commercially available guanidine-thiocyanate–based extraction kit\(^d\) according to the manufacturer’s instructions. Concentration and purity of RNA in each sample were measured via spectrophotometry.\(^1\) Reverse transcription was performed with a kit,\(^1\) and 1 µg of RNA was loaded into each reaction. Complementary deoxyribonucleic acid was then diluted 1:10 with 180 µL of nuclease-free water.

Real-time PCR assays for IL-1\(\beta\), IL-6, IL-8, IL-10, TNF-\(\alpha\), and B2M gene expression were performed as described for whole blood samples. \(\beta\)2 Microglobulin was selected as the housekeeping gene because, in preliminary tests of liver tissue, B2M gene expression was more stable than B-Gus gene expression. Cytokine expression in each sample was normalized to the expression of B2M, and the calibrator used for \(\Delta\Delta\)Ct calculation was each horse’s \(\Delta\Delta\)Ct value after saline solution infusion. Lipopolysaccharide-induced gene expression was therefore expressed relative to the saline solution treatment.

**Statistical analysis**—Mixed-model ANOVA for repeated measures was performed with computer software\(^4\) to determine effects of group (EMS-affected horses vs healthy horses), treatment (LPS vs saline solution), time, and main effect interactions. Area under the curve values for whole blood inflammatory cytokine gene expression were calculated with the trapezoidal method and computer software.\(^3\) Blood and liver tissue gene expression data, AUC for blood gene expression, heart rate, and respiratory rate required logarithmic transformation to fit the ANOVAs normal distribution assumptions. Transformed data are reported as geometric means with 95% CI. All other data are reported as least squares means ± SEM. The autoregressive correlation parameter was included in the final model for analysis of rectal temperature and heart rate only. Mean separation was performed via a Fisher protected least significant difference test, and significance was set at a value of \(P < 0.05\).

Mean cytokine expression at each time point was also compared with the preinfusion baseline via 1-sample \(t\) tests with Bonferroni correction for multiple comparisons. Significance was set at a value of \(P < 0.002\) because the Bonferroni correction was applied. Liver tissue gene expression following LPS administration was compared with findings following saline solution infusion via the same method.

Baseline blood (~30 minutes) and liver tissue (following saline solution administration) gene expressions were compared among groups by recalculating \(\Delta\Delta\)Ct with a pooled calibrator value for each cytokine...
that was generated by calculating the mean of all baseline ∆Ct values for that gene. Mean cytokine expressions were then compared among groups via independent sample t tests.

**Results**

**Clinical signs**—Ten of 12 horses developed transient signs of depression, anorexia, pawing, yawning, head shaking, stretching, or muscle fasciculations following LPS infusion. Two horses (1 healthy horse and 1 horse with EMS from the same pair) had no clinical response to LPS infusion. Physical examination variables did not change from baseline values, and leukopenia was not detected; thus, all data collected from these horses after LPS infusion were excluded from further analyses.

With the exception of the 2 nonresponders, all horses developed fever (rectal temperature > 38.5°C) following LPS infusion. Lipopolysaccharide administration significantly (treatment × time; P < 0.001) increased mean rectal temperature, but no differences were detected among groups (Figure 1). Mean heart rate increased significantly (treatment × time; P < 0.001) following LPS infusion. A group effect was detected for this variable (P = 0.048), with higher heart rates detected in horses with EMS. Mean respiratory rate significantly (treatment × time; P < 0.001) increased following LPS administration, and a group effect revealed higher respiratory rates in horses with EMS (P = 0.017). Group × treatment × time effects were not significant for any of the physical examination variables recorded. Clinical signs of laminitis were not detected in any horse following either LPS or saline solution administration.

**Hematologic and plasma biochemical analyses**—Anemia (RBC count, < 6.3 X 10⁶ cells/µL; reference range, 6.3 X 10⁶ cells/µL to 9.5 X 10⁶ cells/µL) was detected via ≥ 1 CBCs in 5 of 6 horses with EMS and 2 of 6 healthy horses. Concurrently low Hct (< 28%; reference range, 28% to 44%) and hemoglobin concentrations (< 10.2 g/dL; reference range, 10.2 g/dL to 16.0 g/dL) were detected in 5 of 7 horses. Mean ± SEM Hct was significantly lower (P = 0.01) in horses with EMS (32.3 ± 1.0%) than the value for healthy horses (36.6 ± 0.7%). Mean hemoglobin concentration was also lower (P = 0.009) in the EMS-affected horses (10.9 ± 0.3 g/dL) than the value for the healthy horses (12.32 ± 0.2 g/dL).

Leukopenia (total WBC count, < 4.6 X 10³ cells/µL; reference range, 4.6 X 10³ cells/µL to 12 X 10³ cells/µL) was detected 180 minutes after LPS infusion in 9 of 10 LPS responders. Neutropenia (< 2.6 X 10³ cells/µL; reference range, 2.6 X 10³ cells/µL to 5.3 X 10³ cells/µL) and lymphopenia (< 1.5 X 10³ cells/µL; reference range, 1.5 X 10³ cells/µL to 4.3 X 10³ cells/µL) were detected in 7 of 9 and 8 of 9 of those horses, respectively.
Whole blood cytokine gene expressions—in whole blood samples, treatment × time effects were significant for gene expressions of IL-1β (P < 0.001; Figure 2) and TNF-α (P < 0.001), and significant treatment effects were detected for gene expressions of IL-6 (P < 0.001), IL-8 (P < 0.001), and IL-10 (P < 0.001).

Group × treatment × time effects did not reach significance for any of the inflammatory cytokine gene expressions evaluated, but group × time effects were significant (P = 0.027) for whole blood gene expression of IL-1β, and group effects were significant (P = 0.040) for whole blood gene expression of TNF-α.

Figure 2—Fold changes in whole blood gene expression of IL-1β (A), TNF-α (B), IL-6 (C), IL-8 (D), and IL-10 (E) in 6 HHs and 6 EMSHs following IV bolus administration of LPS (20 ng/kg [in 60 mL of sterile SS]) or SS, followed by the other treatment after a 7-day washout period. Two horses (1 HH and 1 EMSH) had no clinical response to LPS infusion, and those data were not used in the analyses. Baseline gene expression (determined 30 minutes prior to infusion [which concluded at 0 minutes]) was equal to a 1-fold change for all cytokines; data are expressed as geometric means and 95% CI and are displayed on a logarithmic scale. Significance was set at a value of P < 0.002 for 1-sample t-test comparisons of cytokine expression at each time point, compared with the baseline value. Significance was set at a value of P < 0.05 for ANOVA main effects and interactions. For gene expressions of IL-1β, treatment × time effects (P < 0.001) and group × time effects (P = 0.027) were detected. For gene expression of TNF-α, treatment × time effects (P < 0.001) and group effects (P = 0.040) were detected. For gene expressions of IL-6, IL-8, and IL-10, treatment effects (P < 0.001) were detected. *Value following LPS infusion is significantly different from baseline value in EMS-affected group. †Value following LPS infusion is significantly different from baseline value in both healthy and EMS-affected groups. See Figure 1 for key.
Results of 1-sample t tests indicated that cytokine expressions remained significantly (P < 0.002) increased from baseline values for longer periods in horses with EMS following LPS administration, compared with healthy horses. Baseline (–30 minute) gene expressions did not differ between healthy horses and horses with EMS.

Lipopolysaccharide administration significantly (treatment, P < 0.001) increased AUC values for IL-1β, IL-6, IL-8, IL-10, and TNF-α whole blood gene expressions, but differences between EMS-affected and healthy horses were not detected via ANOVA (Table 1).

Area under the curve for whole blood gene expression of IL-10 was significantly (group, P = 0.046) increased in horses with EMS, compared with findings in healthy horses.

Liver tissue cytokine gene expressions—Following LPS infusion, gene expression of IL-6 in liver tissue increased (P < 0.025) from values after saline solution infusion in healthy horses, and gene expression of IL-8 increased (P < 0.025) in both healthy and EMS-affected horses (Figure 3). Gene expression of IL-1β, IL-6, IL-8, IL-10, and TNF-α following LPS infusion did not differ in magnitude between the EMS-affected and healthy horses. Baseline gene expression (assessed after saline solution infusion) of IL-1β, IL-6, IL-8, IL-10, and TNF-α did not differ significantly between horses with EMS and healthy horses.

Histologic examination of liver biopsy specimens—Twenty-three biopsy specimens of diagnostic
quality were obtained from the 12 horses. Following LPS infusion, an unusable specimen was collected from 1 horse with EMS; the amount of liver tissue in the specimen was insufficient for analysis. The histologic appearance of tissue specimens did not differ between healthy horses and horses with EMS or between horses that received LPS or saline solution. For specimens collected during the second week of the experimental period, no effects of previous biopsy procedures were observed either via ultrasonography of the liver during the biopsy procedure or on histologic examination.

Discussion

In the present study, systemic inflammation developed in both healthy horses and horses with EMS following an endotoxin challenge, and increases in whole blood inflammatory cytokine gene expressions were detected in both groups. Although the magnitude of the increase in blood gene expression of all evaluated cytokines did not differ between those groups, the duration of the increased IL-6, IL-8, IL-10, and TNF-α expressions was longer for horses with EMS. Endotoxin infusion also increased liver tissue cytokine gene expression, but differences were not detected between healthy horses and horses with EMS. Results of the present study therefore support our hypothesis that EMS affects horses’ inflammatory responses to endotoxin.

Lipopolysaccharide infusion increased rectal temperature, heart rate, and respiratory rate in a manner consistent with findings of previous studies24,25 in horses, but responses did not differ significantly between healthy horses and horses with EMS. These alterations in physical examination variables were expected, considering the known effects of inflammatory mediators such as IL-1β on body temperature regulation and metabolic rate.26 Interestingly, horses with EMS had higher overall heart rates and respiratory rates than did healthy horses following either LPS or saline solution administration. Cardiopulmonary abnormalities relating to fat mass in obese humans are well described,27 and findings from the present study suggested that similar abnormalities occur in obese horses.

In the present study, inflammatory cytokine expressions increased in both blood and liver tissue samples following LPS infusion. Lipopolysaccharide is recognized by the cell-surface pattern recognition receptor TLR4, and many cells, especially those with immunologic functions, express this receptor and respond to LPS stimulation.28 Activation of TLR4 signaling culminates in the secretion of inflammatory mediators that orchestrate the innate and adaptive immune responses. The proinflammatory cytokines IL-1β, IL-6, IL-8, and TNF-α assessed in the present study are major regulators of innate immunity,29 and their gene and protein expressions are known to increase in response to LPS administration in horses.30–33 Interleukin-1β, IL-6, and IL-8 are used as prognostic indicators in humans with sepsis, and increases in expressions of those cytokines predict organ failure and death.34–36 In horses, laminar tissue expression of these mediators increases during the onset of laminitis37; compared with tissues from nonlaminic controls, expressions of IL-1β, IL-6, IL-8, and TNF-α increase in the liver and lungs of laminitic horses.38 Interleukin-10 is an anti-inflammatory mediator that is responsible for limiting inflammation and reducing tissue damage during sepsis.39 When produced in excess, however, IL-10 contributes to the development of immunosuppression, and high IL-10 concentrations are a negative prognostic indicator in humans with sepsis.40 In the present study, cytokine expression was measured at the level of mRNA rather than at the level of protein. Currently, few equine-specific cytokine assays are available, which limits accurate quantification of cytokine proteins. However, regulation of most inflammatory cytokines occurs at the transcriptional level or through posttranscriptional mechanisms involving mRNA stability.41,42 Measurement of mRNA concentration is therefore reflective of protein concentration.

In whole blood samples collected from horses following LPS administration in the present study, the expressions of all evaluated cytokines were increased, compared with baseline values. Gene expression of TNF-α peaked at 60 and 90 minutes in healthy horses and horses with EMS, respectively, and gene expressions of IL-1β, IL-6, IL-8, and IL-10 peaked between 120 and 240 minutes in all horses. These findings are consistent with those of a recent study30 in which IV administration of a bolus of LPS (30 ng/kg) increased inflammatory cytokine gene expression in blood samples collected from healthy horses. However, the times to peak cytokine expression were comparatively longer in the present study; the fact that LPS was administered at a lower dose could account for this discrepancy between results of the 2 studies.

In the present study, the magnitude of cytokine responses did not differ significantly between healthy horses and horses with EMS, but inflammatory gene expression remained increased from baseline for a longer period in horses with EMS. Most notably, TNF-α expression only increased (relative to baseline value) at 1 time point in healthy horses, whereas it was consistently increased in horses with EMS. Groups also differed with respect to expressions of IL-6, IL-8, and IL-10. These differences between healthy horses and horses with EMS are not directly explained by obesity because only 2 of 6 affected horses had generalized obesity, and another 2 horses had regional adiposity. It must therefore be concluded that the historical problem of obesity is not directly explained by obesity because only 2 of 6 affected horses had generalized obesity, and another 2 horses had regional adiposity. It must therefore be concluded that the historical problem of obesity or insulin resistance affected the systemic inflammatory response. It is possible that adipose tissues are abnormal in lean horses with EMS and may contribute to differences in the inflammatory response.

Whole blood cytokine expressions in healthy horses and horses with EMS did not differ before LPS or saline solution infusion, but expressions of IL-1β and TNF-α were higher overall in horses with EMS during the 240-minute period that followed either infusion. Area under the curve for IL-10 gene expression was also higher in horses with EMS. These results further support obesity as a proinflammatory state in horses, as it is in other species, and are consistent with previous findings36 in obese horses. Although increased expression of IL-10 is not consistently associated with obesity, increased IL-10 production by adipose tissues and...
higher blood concentrations in obese humans and rodents, compared with findings for nonobese individuals, have been described.\textsuperscript{43,44} The extent to which obesity impacts circulating leukocyte responses to inflammatory stimuli is not clear. Cross-talk between adipose tissue macrophages and circulating leukocytes is likely to occur because PMBCs from obese humans are in a proinflammatory state and have increased basal cytokine expression, compared with individuals of appropriate body mass index.\textsuperscript{45} Similarly, PMBCs harvested from obese aged horses have different cytokine responses to in vitro stimulation, compared with responses of PMBCs harvested from thin, aged horses,\textsuperscript{46} although this has not been a consistent finding.\textsuperscript{47} Basal PMBC cytokine expression in obese horses has been reported to be the same or even lower than that in clinically normal animals; however, neutrophil oxidative burst activity following phagocytosis is significantly higher in obese animals.\textsuperscript{48}

Potential hormonal modulators of cross-talk between adipose depots and circulating leukocytes include the adipokines adiponectin and leptin. Immune functions consume a substantial amount of energy; thus, adipokines ensure that the immune system remains apprised of available energy stores in the body.\textsuperscript{49} Adiponectin, which decreases with both obesity\textsuperscript{49} and inflammation,\textsuperscript{50} exerts direct anti-inflammatory effects on cells of the immune system.\textsuperscript{1} Leptin secretion increases in direct proportion to fat mass\textsuperscript{49} and also in response to inflammation.\textsuperscript{51} Leptin promotes proinflammatory responses such as cytotoxic polarization of lymphocyte differentiation and increases in inflammatory cytokine expressions.\textsuperscript{3,10} Whether obesity affects leukocyte responses to endotoxin in vivo remains unknown, but results of the present study have suggested that cytokine production is prolonged in horses with EMS.

In the present study, we did not demonstrate that EMS altered the magnitude of blood inflammatory cytokine responses in horses, but it is possible that exaggerated inflammatory responses occur in tissues rather than in circulating leukocytes. Adipocytes express TLR4 and are capable of responding to LPS stimulation by secreting proinflammatory cytokines and chemokines such as IL-6, TNF-\(\alpha\), and macrophage chemotactrant protein-1.\textsuperscript{3,2,5} Cultured equine adipocytes and preadipocytes produce IL-1\(\beta\), IL-6, and TNF-\(\alpha\) in response to LPS stimulation, and adipose tissue biopsy specimens obtained from horses following IV LPS infusion have increased expression of these cytokines, compared with findings following IV administration of saline solution.\textsuperscript{53} Inflammatory reactions within tissues in horses with EMS may therefore be exaggerated by virtue of increased fat mass or abnormal adipose tissues.\textsuperscript{32} Adipose tissue cytokine responses were not assessed in the horses used in the present study but warrant further consideration.

Lipopolysaccharide administration increased the expression of IL-6 and IL-8 in liver tissues, but differences in those gene expressions were not apparent between healthy horses and horses with EMS in the present study. It is possible that aspects of the study design limited our ability to detect differences between groups. Because liver tissue cytokine responses to an endotoxin challenge have not previously been examined in horses, to our knowledge, timing of sample collection was based on gene expression studies in rodents\textsuperscript{49} and approximately reported increases in equine serum and plasma cytokine expressions after IV administration of LPS.\textsuperscript{31,32} Differences in gene expressions between healthy horses and horses with EMS might have been detected had the timing of sample collection been different or multiple samples been collected. In other species, obesity promotes hepatic inflammation because intermediates of lipid metabolism are toxic to hepatocytes and activate Kupffer cells.\textsuperscript{19} Lipid accumulation within hepatocytes also distorts sinusoids and decreases blood flow velocity, leading to prolonged exposure of Kupffer cells to antigens or leukocytes.\textsuperscript{18} Liver dysfunction might also reduce clearance of toxins that escape the gastrointestinal tract during minor intestinal carbohydrate overload events that occur in horses and ponies while grazing on pasture, exposing affected animals to more frequent and severe inflammatory events and increasing their risk of developing laminitis.\textsuperscript{14} More comprehensive examination of liver dysfunction in horses with EMS is indicated in future studies.

An important finding of the present study is that IV administration of LPS neither induced laminitis in healthy horses nor exacerbated the condition in horses with EMS. Although horses with clinical signs suggestive of endotoxemia are at increased risk of developing laminitis\textsuperscript{30} and circulating endotoxin has been detected in horses during carbohydrate overload laminitis induction,\textsuperscript{46} administration of LPS alone has consistently failed to induce the disease. Endotoxemia may act synergistically with other factors such as hindgut-derived gram-positive exotoxins or vasoactive amines\textsuperscript{57} to induce laminitis, but multiple factor models have not been explored in horses to our knowledge.

One limitation of the present study may have been that the horses with EMS were part of an intensively managed research herd. It is therefore possible that had LPS been administered to obese horses that were in an exacerbated state of inflammation and insulin resistance, greater differences in the cytokine responses between groups would have been revealed. Lean body condition scores could explain why baseline whole blood or liver tissue gene expression values and histopathologic findings in liver tissue specimens did not differ between healthy horses and horses with EMS. Compared with healthy horses, horses with EMS responded differently to endotoxin challenge, even when they had a lean phenotype. This difference could reflect underlying physiologic defects that were not directly related to adiposity or indicate that adiposity in horses with EMS was not adequately assessed by body condition score. Results from a study\textsuperscript{48} in obese humans indicate that metabolic variables can remain abnormal following weight loss. In the present study, some horses with EMS that had a lean phenotype retained abnormal characteristics such as regional adiposity. Additionally, certain phenotypic abnormalities in horses with EMS may only be unmasked in response to a challenge. For example, laminitis-prone obese ponies develop sea-
sonal hypertension and reduced insulin sensitivity in response to high dietary carbohydrate intake.\textsuperscript{39} Therefore, endotoxin challenge may have unmasked inherent abnormalities in the inflammatory responses of horses with EMS in the present study. An interesting finding in the present study was that anemia was detected at least once in 5 of the 6 horses with EMS, and EMS-affected horses had significantly lower Hct and hemoglobin concentrations than did healthy horses. Horses with EMS may develop anemia of chronic disease as a result of obesity-related inflammation.\textsuperscript{60} Iron deficiencies in obese humans\textsuperscript{61} have been described and are ascribed to both true iron deficiency and inflammation-related functional deficiencies.\textsuperscript{62} Iron status was not assessed in the horses in the present study, but measurements of serum iron and ferritin concentrations and total iron binding capacity should be considered in similar experiments in the future.

Results of the present study have led us to conclude that EMS affects horses’ systemic inflammatory responses to endotoxin by prolonging inflammatory cytokine gene expression in blood. However, the magnitude of the inflammatory response does not differ between horses that are healthy and those with EMS. Intravenous administration of LPS also induced proinflammatory cytokine expression in liver tissues of both healthy horses and horses with EMS. Notably, endotoxin challenge did not induce laminitis or exacerbate the condition in horses with EMS. Further research is required to determine the role of inflammation in horses with EMS. Future studies should also include direct assessment of adipose tissue inflammatory responses.

References