# Effects of infusion of adenosine triphosphate-magnesium chloride on cardiopulmonary and clinicopathologic variables, cytokine activity, and endothelin concentration in horses administered a low dose of endotoxin

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**Objective**—To evaluate systemic effects of IV infusion of ATP-$MgCl_2$ subsequent to infusion of a low dose of endotoxin in horses.

**Animals**—12 adult horses.

**Procedure**—Horses were administered endotoxin (lipopolysaccharide [LPS]) or saline (0.9% NaCl) solution, IV, during a 30-minute period. Immediately thereafter, horses in each group were infused IV with ATP-$MgCl_2$ or saline solution. Two weeks later, horses were administered the opposite solution (LPS or saline solution), but it was followed by the same infusion as 2 weeks previously (ie, ATP-$MgCl_2$ or saline solution). Cardiopulmonary and clinicopathologic variables, cytokine activity, and endothelin (ET) concentrations were recorded.

**Results**—IV infusion of ATP-$MgCl_2$ after administration of a low dose of endotoxin failed to attenuate the cardiopulmonary, clinicopathologic, and cytokine alterations that develop secondary to endotoxin exposure. The combination of LPS and ATP-$MgCl_2$ potentiated pulmonary hypertension, leukopenia, and neutropenia when compared with the combination of LPS and saline solution. The combination of LPS and ATP-$MgCl_2$ resulted in thrombocytopenia. Endothelin concentration was increased in jugular venous and pulmonary arterial plasma in horses receiving LPS and ATP-$MgCl_2$. Similar increases were not observed with LPS and saline solution.

**Conclusions and Clinical Relevance**—Administration of ATP-$MgCl_2$ did not protect horses from systemic effects of experimentally induced endotoxemia. Furthermore, the use of ATP-$MgCl_2$ during endotoxemia may worsen the cardiopulmonary and clinicopathologic status of affected horses. Because ATP and other adenine nucleotides are released from cells during shock, their potential role in the development of hemodynamic derangements, leukocyte adherence, and coagulopathies during endotoxemic episodes warrants further investigation. (*Am J Vet Res* 2004;65: 225–237)

Endotoxemia remains the leading cause of death in horses.[1,2] Endotoxemia affects all horses regardless of age, breed, sex, or geographic location. The prevalence of endotoxemia in horses with acute gastrointestinal tract disease (ie, colic) admitted to referral veterinary hospitals is estimated to be approximately 25%, and it represents a major cause of death in horses with colic.[1,3,4] The most common gastrointestinal tract diseases associated with endotoxemia include enterocolitis-enteritis and intestinal strangulation-obstruction and are often associated with complications such as laminitis and gastrointestinal ileus.[5]

Several inherent mechanisms restrict transmural movement of endotoxins and bacteria from the lumen of the gastrointestinal tract, including epithelial cells and intercellular tight junctions, cellular secretions, and the lamina propria.[2] Disruption of the mucosal barrier of the intestines allows transmural passage of endotoxins into the systemic circulation. When a sufficient amount of endotoxin enters the portal circulation, the ability of the liver to remove it may be overwhelmed, resulting in systemic endotoxemia.[6] In comparison with other species, horses are exquisitely sensitive to the effects of endotoxin.[7]

Experimental methods used for the study of endotoxemia can induce clinicopathologic alterations that parallel changes during naturally acquired endotoxemia.[7-9] In a study[9] that used IV administration of a dose of endotoxin (35 ng/kg), investigators observed a marked, acute inflammatory response. Increases in serum activity of inflammatory cytokines were evident, as well as alterations in clinical signs and hematologic variables, and these changes mimicked naturally acquired disease.[9]

Bacteremia attributable to gram-negative organisms or endotoxemia causes decreases in cardiac output, systemic vascular resistance, and mean arterial

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pressure, whereas there is an increase in pulmonary vascular resistance.[10,11] Some of the hemodynamic derangements during endotoxemia may be subsequent to release of **endothelin-1 (ET-1)**, the most potent vasoconstrictor of mammalian vessels that has been identified.[12] Concentrations of ET-1 increase during endotoxemia secondary to endotoxin-induced stimulation of endothelial cells.[13] Endothelin-1 may act as a local vasoconstrictor during severe endotoxic shock and contribute to pulmonary hypertension and peripheral vasomotor disturbances (eg, decreased blood flow to the gastrointestinal tract and digits) observed during this syndrome.[13,14] Analysis of results of studies[15-22] to evaluate the efficacy of a combination of ATP-$MgCl_2$ suggests that this product has potential beneficial effects in patients with hypoperfusion (ie, low-flow conditions) or organ ischemia. The use of ATP-$MgCl_2$ following hemorrhagic shock and other adverse circulatory conditions in humans and laboratory animals can improve mitochondrial function and tissue ATP content[15,16]; restore organ function, blood flow, and perfusion[15-19]; improve reticuloendothelial function, survival time, and survival rate[20,21]; and downregulate the synthesis and release of inflammatory cytokines (eg, **tumor necrosis factor-α [TNF-α]** and **interleukin [IL]**-6).[22]

In a study[23] conducted by our laboratory group, IV administration of ATP-$MgCl_2$ to healthy conscious horses was associated with a rate-dependent increase in cardiac output, decrease in systemic vascular resistance, and mild pulmonary hypertension without any appreciable detrimental effects. In another study[24] conducted by our laboratory group, IV infusion of ATP-$MgCl_2$ at a rate of 0.3 mg of ATP/kg/min to healthy anesthetized horses caused a significant decrease in colonic vascular resistance without a corresponding decrease in colonic arterial blood flow. Analysis of these results suggests that IV infusion of ATP-$MgCl_2$ could have beneficial effects during low-flow conditions by improving tissue perfusion and providing an energy substrate (ie, ATP) directly to ischemic tissues for use in maintenance of cellular metabolism.

Administration of ATP-$MgCl_2$, which has vasodilatory actions, increases cardiac output, and delivers an energy substrate directly to the tissues, offers a potential treatment for horses with gastrointestinal tract ischemia and endotoxic shock. The purpose of the study reported here was to evaluate the systemic effects of IV infusion of ATP-$MgCl_2$ after low-dose endotoxin infusion in horses. We hypothesized that IV infusion of ATP-$MgCl_2$ would attenuate the pathophysiologic alterations of clinical signs; hemodynamic, metabolic, hematologic, and serum biochemical variables; activity of TNF-α and IL-6; and ET-1 concentrations subsequent to infusion of a low dose of endotoxin in clinically normal, conscious adult horses.

## Materials and Methods

**Horses**—Twelve clinically normal horses (8 Thoroughbreds and 4 Quarter Horses; 8 castrated males and 4 sexually intact females) that ranged from 6 to 15 years of age (median, 13 years) and weighed 465.9 to 604.5 kg (median, 505.7 kg) were included in the study. Horses were maintained on a routine preventive health care program. Horses were housed separately in box stalls and allowed to acclimate for a minimum of 10 days prior to the start of the study. The study was approved by the Institutional Animal Care and Use Committee of Louisiana State University.

**Instrumentation**—Instruments were inserted in each horse by use of described techniques.[23,24] For placement of all catheters, the overlying skin was aseptically prepared, subcutaneous tissues were infiltrated with lidocaine, and catheters were inserted percutaneously. A 14-gauge, 5.1-cm fluoride-coated catheter[a] was inserted into the left jugular vein for infusion of the pretreatment solution of endotoxin (**lipopolysaccharide [LPS]**) or saline (0.9 % NaCl) solution and the treatment solutions (saline solution or ATP-$MgCl_2$). Polyethylene tubing[b] (**outside diameter [OD]**, 1.57 mm) was inserted distal to the first catheter and advanced until the tip was positioned in the right atrium for determination of **mean right atrial pressure (MRAP)**. A balloon-tipped, flow-directed thermodilution catheter[c] that was used to measure **cardiac output (CO)** and **pulmonary arterial pressure (PAP)** was inserted distal to the first 2 catheters and advanced until the distal port was positioned in the pulmonary artery. A 14-gauge, 13.3-cm fluoride-coated catheter[d] was inserted into the right jugular vein for collection of venous blood samples. Polyethylene tubing[e] (OD, 1.77 mm) was inserted distal to the first catheter and advanced until the tip was positioned in the right ventricle for infusion of ice-cold polyionic fluids[f] for measurement of CO. A 55-mL volume of fluid was infused into the right ventricle during a 4-second period by use of a $CO_2$-driven injector,[g] and the CO was derived on the basis of thermodilution.[f,g] A second piece of polyethylene tubing (OD, 1.77 mm) was inserted distal to the first piece of polyethylene tubing and advanced until the tip was positioned in the pulmonary artery for collection of blood samples. Arterial blood pressure was measured by use of a 20-gauge, 4.45-cm polyurethane-coated catheter[h] placed in the transverse facial artery. All catheter positions were confirmed on the basis of characteristic pressure waveforms. All pressure transducers[i] were positioned at the height of the point of the shoulder. Pressure transducers and CO meter[j] were connected to a polygraph,[k] and pressure and CO curves were recorded on a chart recorder.[l] A continuous base-apex ECG also was obtained.

**Experimental design**—During the experiments, horses were cross-tied in stalls (1.82 × 1.82 m), and hay and water were provided ad libitum. Horses were administered endotoxin (*Escherichia coli* 055:B5[m]; 35 ng/kg, IV) or an equivalent volume of saline solution by use of an infusion pump[n] during a 30-minute period. Horses were randomly assigned to the pretreatment infusion group. Horses in each pretreatment group were then randomly assigned to 1 of 2 treatment groups (saline solution or ATP-$MgCl_2$[o]). Immediately after completion of the pretreatment infusion, horses were administered the assigned treatment infusion (100 µmol ATP/kg and 100 µmol $MgCl_2$/kg at an infusion rate of 0.3 mg of ATP/kg/min or an equivalent volume of saline solution infused during the same period). The formulation of ATP-$MgCl_2$ has been described elsewhere.[23,25] Two weeks later, horses received the opposite pretreatment solution (ie, horses that had been administered LPS were administered saline solution and vice-versa) but were administered the same treatment infusion (ie, ATP-$MgCl_2$ or saline solution) that had been infused 2 weeks previously. The 4 groups were **saline-saline (SS)**, **LPS-saline (LS)**, **saline-ATP-$MgCl_2$ (SA)**, and **LPS-ATP-$MgCl_2$ (LA)**.

Clinical signs and hemodynamic variables were determined 0.5 hours prior to the start of the pretreatment infusion (baseline; –0.5 hours), at the end of the pretreatment infusion (0 hours), 5 minutes after starting the treatment infusion, and at 30-minute intervals for 6 hours after onset of the treatment infusion. Arterial blood gas analyses were

determined at –0.5, 0, 0.5, 1, 1.5, 2, 4, 6, 8, and 12 hours. Complete blood counts were determined at –0.5, 0.5, 1, 1.5, 2, 4, 6, 8, 12, 18, and 24 hours. Serum biochemical analyses data were determined at –0.5, 2, 6, 12, 18, and 24 hours. Serum activity of cytokines (TNF-α and IL-6) was determined prior to insertion of instruments and at –0.5, 0, 0.5, 1, 1.5, 2, 4, 6, 8, 12, 18, and 24 hours. Endothelin-1 concentrations were determined prior to insertion of instruments (samples obtained only from the jugular vein) and then at the same time points as for serum cytokines (samples obtained from the jugular vein and pulmonary artery). Instruments were removed from all horses at completion of the experiment (ie, at 6 hours), except for the catheters in the transverse facial and pulmonary arteries, which were removed at 12 hours, and a catheter in 1 of the jugular veins, which was removed at 24 hours.

**Clinical signs of disease**—Horses were monitored throughout the experimental period. Heart rate, respiratory rate, rectal temperature, mucous membrane color, capillary refill time, and behavior were recorded.

**Hemodynamic variables**—Hemodynamic variables that were measured included systolic arterial pressure, diastolic arterial pressure, **mean arterial pressure (MAP)**, systolic PAP, diastolic PAP, **mean PAP (MPAP)**, MRAP, and CO. Three measurements were obtained at each time point for each pressure variable. Five measurements were obtained for CO at each time point, and the 3 middle values were used in the analyses. Cardiac index was calculated as CO divided by body weight. **Systemic vascular resistance ($SR_I$)** was calculated as (MAP – MRAP)/CO. **Pulmonary vascular resistance ($PR_I$)** was calculated as MPAP divided by CO.[26] Specific ECG alterations were recorded.

**Metabolic variables**—Heparinized blood samples (2 mL/sample) were collected anaerobically from the transverse facial artery and stored on ice until analyzed[p] for pH, $Paco_2$, $Pao_2$, **arterial oxygen saturation ($Sao_2$)**, bicarbonate concentration, total $CO_2$, and base excess. All samples were analyzed within 10 minutes after collection. **Systemic arterial oxygen content ($Cao_2$)** was calculated as the sum of oxygen bound to hemoglobin and oxygen dissolved in plasma by use of the following equation[23]:

$$Cao_2 = ([\text{Hemoglobin} \times \text{percentage } Sao_2 \times 1.34] + [Pao_2 \times 0.003])$$

Oxygen delivery was estimated as the product of $Cao_2$ times CO.[23]

**Hematologic variables**—Blood samples (3 mL/sample) were collected from a jugular vein into tubes containing EDTA. Samples were analyzed to determine PCV, concentration of total solids, results of a CBC[q] and WBC differential cell count, and fibrinogen concentration.

**Serum biochemical analyses**—Blood samples (6 mL/sample) were collected from a jugular vein into tubes containing lithium heparin, and plasma as analyzed[r] to measure glucose, total bilirubin, albumin, globulin, BUN, creatinine, calcium, phosphorus, sodium, potassium, chloride, and magnesium concentrations and activity of aspartate transaminase, γ-glutamyltransferase, **alkaline phosphatase (ALP)**, and **creatine kinase (CK)**. The anion gap was calculated.

**Serum cytokine bioassays**—Blood samples (10 mL/sample) were collected from a jugular vein into clot tubes. After blood clots formed, tubes were centrifuged at 1,500 × *g* for 10 minutes, and serum was harvested. Serum samples were placed into 4 tubes and stored at –70°C until analyzed for cytokine activity (IL-6 and TNF-α) by use of bioassays.[9]

Cell lines were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 2.2mM L-glutamine, 2.2 g of $NaHCO_3$/L, 25mM HEPES, $2.7 \times 10^{-4}$ mg of 2-mercaptoethanol/L, 100 U of penicillin/mL, and 100 µg of streptomycin sulfate/mL.[9] In addition, B13.29 clone B9 cells were supplemented with 50 U of recombinant murine IL-6[s]/mL. Equine cytokine standards were generated by culturing $3 \times 10^6$ equine peripheral blood mononuclear cells/mL with 5 µg of bacterial LPS/mL in 5% $CO_2$ for 6 hours at 39°C. Cultures were centrifuged, the supernatant filtered, and aliquots frozen at –20°C.[8,27-30]

Because the dose-response curves for recombinant human and murine cytokines were not parallel with those obtained for equine standards, the recombinant cytokines were used as a reference standard to determine units for the equine cytokine standards in both bioassays. Equine cytokine standards were serially diluted 5-fold and 3-fold for the TNF-α and IL-6 assays, respectively. The recombinant cytokines were added to the plates at 3 concentrations (10, 1, and 0.1 U/mL). Values obtained for the recombinant cytokines were then used as a reference to determine the number of units of cytokine per milliliter in the equine standards.

A cytotoxicity assay that used mouse fibrosarcoma cell line WEHI 164 clone 13 cells[9] was performed to measure activity of TNF-α. Briefly, 50 µL of test supernatant (1:50 dilution) was pipetted in triplicate into 96-well, flat-bottomed, microtitration plates.[t] Next, $2 \times 10^4$ WEHI 164 clone 13 cells were added to each well in 50 µL of complete medium containing 2 µg of actinomycin D[u]/mL, which resulted in a final concentration of 1 µg of actinomycin D/mL. Plates were sealed and incubated in 5% $CO_2$ for 20 hours at 37°C. Ten microliters of a solution of tetrazolium dye (3-[4,5 dimethylthiazole-2yl]-2,5 triphenyl tetrazolium bromide[v]; 5 mg/mL in calcium magnesium-free PBS solution) was added to each well. After incubation for 4 hours at 37°C, 100 µL of 0.04 N HCl in isopropanol was added to each well, and the contents were pipetted vigorously to dissolve purple formazan crystals. Values were measured at 570 nm in a multiple-well scanning spectrophotometer.[8,w] Sensitivity of the TNF-α assay was 0.04 U/mL.

Serum IL-6 activity was measured by use of murine hybridoma IL-6 dependent cell line B13.29 clone B9 cells. Briefly, test samples were diluted 1:50 in culture medium, and 100 µL was placed in each well of a 96-well, flat-bottomed, microtitration plate. Two thousand B9 cells in 100 µL of medium were seeded into each well and incubated in 5% $CO_2$ for 72 hours at 37°C. During the last 8 hours of incubation, plates were pulsed with 1.0 µCi of [$^3$H] thymidine/well. Proliferation was determined by harvesting the cells and determining [$^3$H] thymidine incorporation by use of liquid scintillation counting.[8,28,29,x] Sensitivity of the assay for IL-6 was 1.677 U/mL.

**Assay of ET-1 concentration**—Blood samples (7 mL) were obtained from a jugular vein and pulmonary artery; each sample was collected in polypropylene tubes containing 105 µL of EDTA and 370 µL of aprotonin.[y] Blood samples were centrifuged at 1,500 × *g* for 10 minutes. Plasma samples were placed into 4 tubes and stored at –70°C until analyzed for ET-1 concentrations by use of a commercial human ELISA kit.[z]

Briefly, 1 mL of sample was mixed with 1.5 mL of precipitating agent. Samples were cooled to 4°C and centrifuged for 20 minutes at 3,000 × *g*. Supernatant was transferred to another polypropylene tube and dried under a stream of nitrogen gas at 37°C. Dried samples were redissolved in 500 µL of assay buffer. Serial dilutions of the ET stock solution were prepared to serve as standards. Buffer was used as the zero standard.

Two hundred microliters of standards, control samples, and unknown samples was pipetted into wells. Detection antibody (50 µL) was added to all wells except the blank well

and mixed appropriately. Wells were covered with plastic film and incubated 16 to 24 hours at 20° to 22°C. Contents of each well were discarded, and wells were washed 5 times with washing buffer. Conjugate (200 µL) was then added to all wells, and the wells were covered with plastic film and incubated for 3 hours at 37°C. Contents of the wells were discarded, and the wells were washed 5 times with washing buffer. Substrate (200 µL) was added to each well, and the wells were incubated in darkness for 30 minutes at 20°C. Then, 50 µL of stop solution was added to each well, and contents were mixed thoroughly. Absorption was determined immediately at 405 nm by use of an ELISA reader,[aa] with values at 620 nm as a reference. All samples were analyzed in duplicate. During the validation procedure, pooled plasma samples from multiple horses were assessed repetitively within an assay and in several assays. Sensitivity of the assay was approximately 1.5 pg/mL. The intra- and interassay variability for equine plasma in our laboratory was 6.4% and 15.4%, respectively. Pooled equine plasma was then spiked with a known quantity of human ET standard; mean recovery of this standard was 102.6%.

**Statistical analysis**—The study was considered a mixed-effect, nested-factorial design with horses nested within treatments. Data were considered continuous and had normal distributions, as determined by use of the Shapiro-Wilk statistic with failure to reject the null hypothesis of normality at $P < 0.05$. Non-normally distributed data were transformed. Data were summarized and graphed as mean ± SEM.

Data were analyzed by use of a mixed-effect general linear model that accounted for the random effect of horse nested within treatments and repeated measurements on each horse. Interaction effects were included. When significant ($P < 0.05$) effects of time and interactions of treatment, endotoxin, and time were detected, predetermined multiple comparisons were conducted by use of adjusted least-squares means and maintenance of an experiment-wise error of $\alpha = 0.05$. Within-group comparisons were made to corresponding values at –0.5 hours to describe changes over time. Treatments that had differing patterns over time were implied to be different. Because SS and SA groups functioned as control groups, between-group comparisons were made for LS and LA groups only when both changed similarly over time from their corresponding values for –0.5 hours. A commercially available statistical program[bb] was used for all analyses.

Cytokine values were considered continuous data, but they did not have a normal distribution, as determined by use of the Shapiro-Wilk test with rejection of the null hypothesis of normality at $P < 0.05$. Transformation of data was unsuccessful. The values were compared among pretreatment groups for differences between treatments over time by use of the Friedman test for comparisons of repeated ranked data. When a significant difference was observed, post hoc testing to deter-

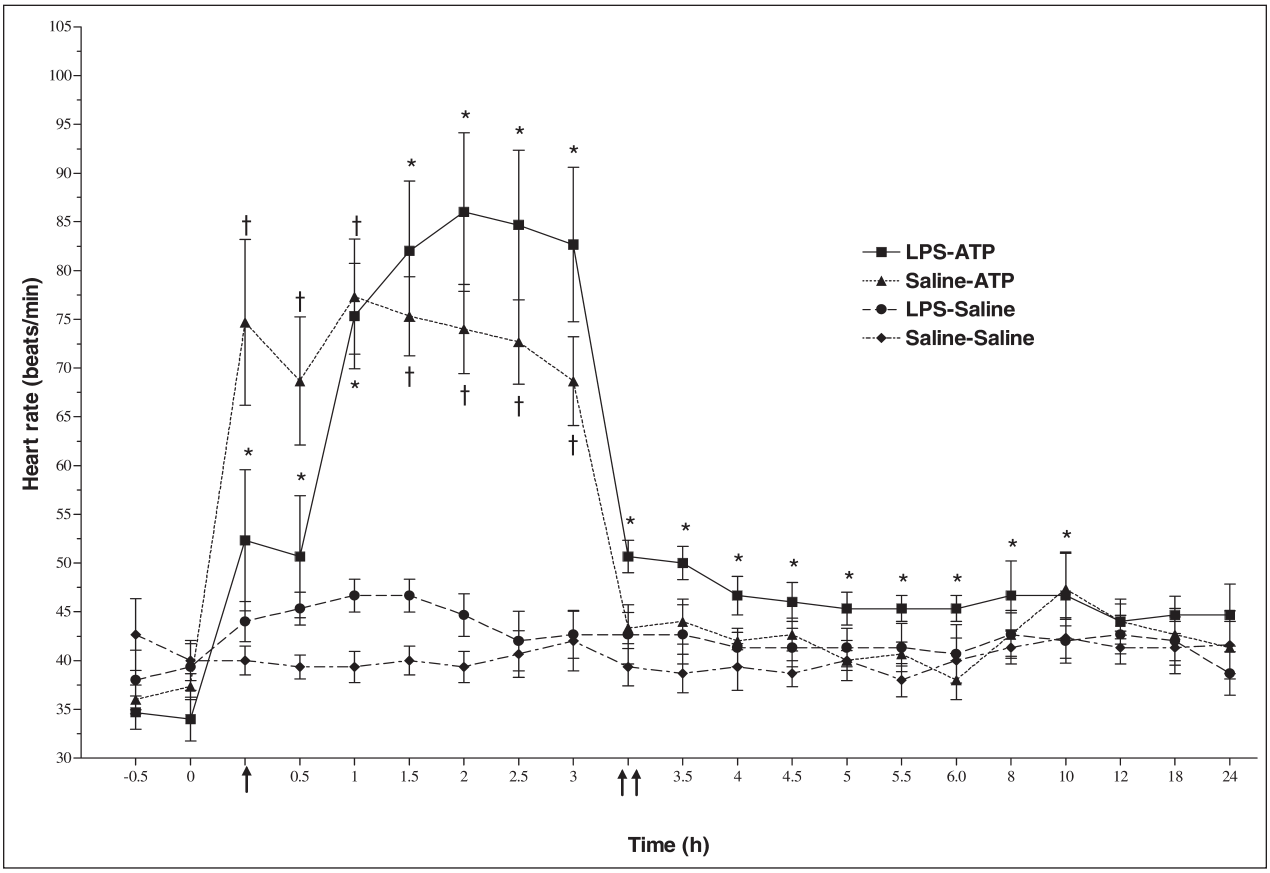



Figure 1—Mean ± SEM heart rate in horses administered a pretreatment infusion of endotoxin (lipopolysaccharide [LPS]; 35 ng/kg during a 30-minute period) or an equivalent volume of saline (0.9% NaCl) solution during the same time period, which was followed immediately by administration of a treatment solution of ATP-$MgCl_2$ (100 µmol ATP/kg and 100 µmol $MgCl_2$/kg at a rate of 0.3 mg of ATP/kg/min) or an equivalent volume of saline solution. The pretreatment infusion was initiated at –0.5 hours. The treatment infusion was initiated at 0 hours and continued for 3 hours. Notice that a sample was obtained 5 minutes after treatment infusion (arrow) and another sample was obtained 5 minutes after discontinuation of the treatment infusion (double arrow). *,†Within a group, value differs significantly ($P < 0.05$) from value for –0.5 hours (*LPS-ATP-$MgCl_2$ [LA]; †saline-ATP-$MgCl_2$ [SA]). Treatment groups that had differing patterns over time were implied to be significant different. Within a specific time point, between-group comparisons (LA and LPS-saline [LS] groups) were only made when both groups were significantly different from their corresponding baseline value.

Table 1—Mean respiratory rate and rectal temperature in horses administered a pretreatment infusion of endotoxin (lipopolysaccharide [LPS]; 35 ng/kg during a 30-minute period) or an equivalent volume of saline (0.9% NaCl) solution during the same time period, which was followed immediately by administration of a treatment solution of ATP-$MgCl_2$ (100 µmol ATP/kg and 100 µmol $MgCl_2$/kg at a rate of 0.3 mg of ATP/kg/min) or an equivalent volume of saline solution

| | Respiratory rate (breaths/min) | | | | Rectal temperature (°C) | | | |
|---|---|---|---|---|---|---|---|---|
| **Time (h)** | **LA** | **SA** | **LS** | **SS** | **LA** | **SA** | **LS** | **SS** |
| –0.5 | 13.33 | 16.00 | 19.33 | 19.33 | 37.75 | 37.88 | 37.67 | 37.93 |
| 0 | 13.33 | 16.00 | 18.00 | 18.67 | 37.92 | 37.78 | 37.78 | 37.97 |
| 5 minutes after starting treatment infusion | 37.33* | 27.33* | 18.67 | 15.33 | 37.93 | 37.88 | 37.83 | 37.88 |
| 0.5 | 30.67* | 21.33 | 18.40 | 17.33 | 38.15 | 37.93 | 37.95 | 37.95 |
| 1.0 | 24.00* | 20.00 | 20.00 | 16.67 | 38.27* | 38.10 | 38.15* | 38.05 |
| 1.5 | 22.67* | 20.67 | 16.67 | 16.00 | 38.58* | 38.28 | 38.53* | 38.07 |
| 2.0 | 22.00 | 24.00 | 16.00 | 16.80 | 39.13* | 38.33 | 39.00* | 38.25 |
| 2.5 | 21.33 | 21.33 | 18.00 | 17.33 | 39.20* | 38.28 | 38.93* | 38.35 |
| 3.0 | 23.33* | 21.33 | 24.00 | 17.33 | 39.30* | 38.35 | 39.12* | 38.25 |
| 5 minutes after stopping treatment infusion | 26.00* | 16.00 | 23.33 | 17.33 | 39.08* | 38.38* | 38.95* | 38.27 |
| 3.5 | 25.33* | 16.00 | 25.33 | 18.00 | 39.07* | 38.32 | 38.90* | 38.32 |
| 4.0 | 27.67* | 16.67 | 22.00 | 18.00 | 38.85* | 38.22 | 38.78* | 38.28 |
| 4.5 | 24.67* | 18.00 | 23.33 | 17.33 | 38.93* | 38.43* | 38.60* | 38.23 |
| 5.0 | 24.67* | 14.67 | 19.33 | 19.33 | 38.63* | 38.43* | 38.55* | 38.12 |
| 5.5 | 22.67* | 16.00 | 20.00 | 16.00 | 38.65* | 38.37* | 38.48* | 38.12 |
| 6.0 | 22.67* | 17.33 | 20.00 | 16.67 | 38.75* | 38.47* | 38.20* | 38.12 |
| 8.0 | 19.33 | 20.00 | 18.00 | 18.00 | 38.68* | 38.43* | 38.33* | 38.38 |
| 10.0 | 18.67 | 20.67 | 14.67 | 17.33 | 38.55* | 38.18 | 38.18* | 38.12 |
| 12.0 | 16.67 | 16.67 | 16.00 | 16.00 | 38.37* | 38.15 | 38.07 | 38.03 |
| 18.0 | 18.67 | 19.33 | 20.00 | 18.67 | 37.70 | 37.83 | 37.78 | 37.98 |
| 24.0 | 19.00 | 19.67 | 23.33 | 18.00 | 37.57 | 37.75 | 37.85 | 37.82 |

*Within a column, value differs significantly ($P < 0.05$) from value at –0.5 hours.
Time –0.5 = Onset of pretreatment infusion. Time 0 = Onset of treatment infusion. LA = LPS-ATP-$MgCl_2$. SA = Saline solution-ATP-$MgCl_2$. LS = LPS-saline solution. SS = Saline solution-saline solution.

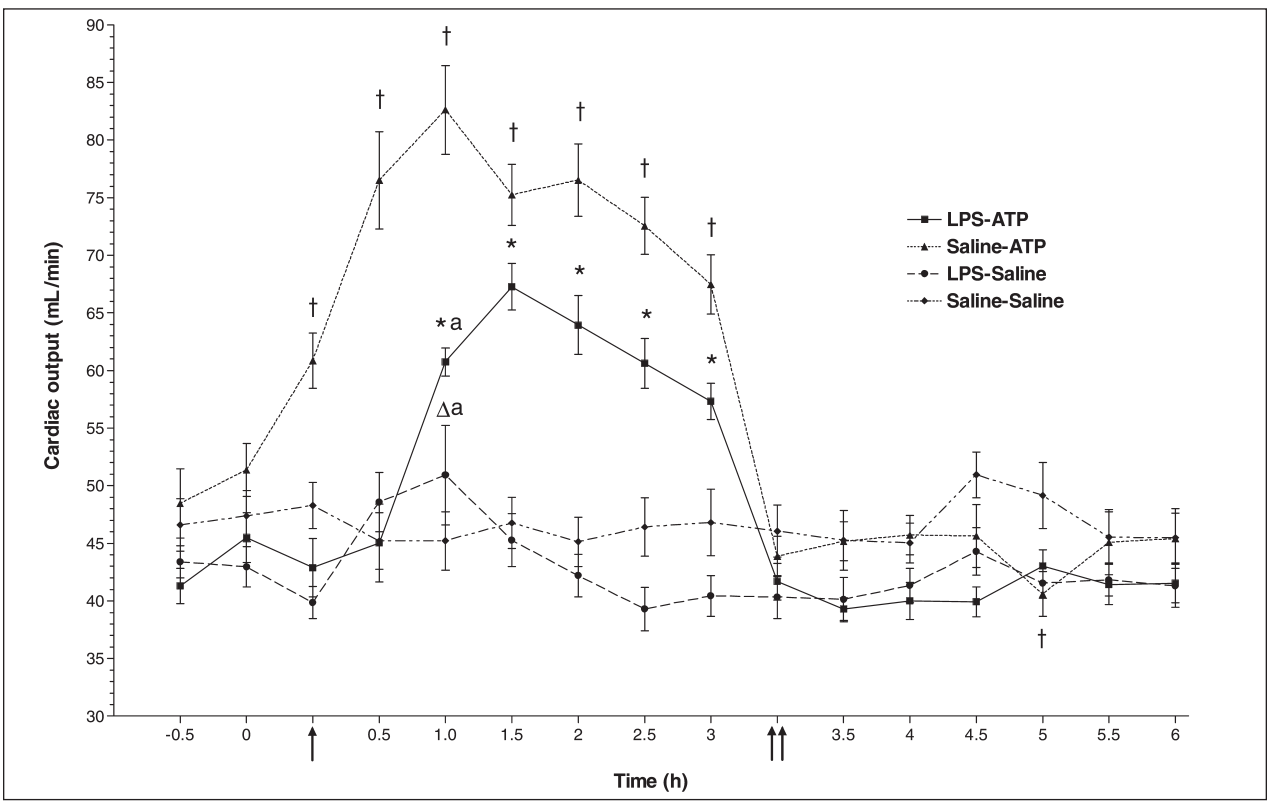



Figure 2—Mean ± SEM cardiac output in horses administered a pretreatment infusion of LPS or an equivalent volume of saline solution, which was followed immediately by administration of a treatment solution of ATP-$MgCl_2$ or an equivalent volume of saline solution. Δ=Within the LS group, value differs significantly ($P < 0.05$) from value for –0.5 hours. a,—Within a specific time point, between-group comparisons (LA and LS groups) were made, but values did not differ significantly ($P < 0.05$) between the LA and LS groups. *See* Figure 1 for remainder of key.

mine the time points at which differences occurred was conducted by use of the method of Rhyne and Steel.[31] Type-I error was maintained at 0.05. Data were summarized as median and range values.

## Results

**Clinical signs of disease**—Heart rate was significantly increased in SA and LA horses (**Fig 1**). Respiratory rate was significantly increased in SA and LA horses (**Table 1**). Rectal temperature was significantly increased in SA, LS, and LA horses. We did not detect significant differences in rectal temperature between LS and LA horses, nor did we detect consistent changes in mucous membrane color and capillary refill time over time for any group. Most of the LS and LA horses had signs of transient abdominal pain with subsequent anorexia and lethargy after LPS administration. These behavioral alterations persisted for approximately 2 hours.

**Hemodynamic variables**—Cardiac output was significantly increased in LA and SA horses over time (**Fig 2**). There was a transient significant increase in LS horses. Cardiac index had a pattern similar to that of CO (data not shown).

The MAP significantly increased in LS and LA horses and decreased in SA horses (**Fig 3**). The systolic arterial pressure and diastolic arterial pressure had patterns similar to that of MAP.

A significant, transient increase in $SR_L$ followed by a decrease during the ATP-$MgCl_2$ infusion was observed in LA horses. Upon discontinuation of the infusion, $SR_L$ increased transiently (**Fig 4**). In SA horses, $SR_L$ significantly decreased during the ATP-$MgCl_2$ infusion. In LS horses, there was a significant, transient increase in $SR_L$.

In LS, LA, and SA horses, MPAP increased significantly (**Fig 5**). In SS horses, MPAP decreased transiently. The systolic PAP and diastolic PAP had patterns similar to that for MPAP.

A significant transient increase in $PR_L$ was detected in the LA, LS, and SA groups (**Fig 6**). In LA and SA horses, $PR_L$ significantly decreased toward the end of the ATP-$MgCl_2$ infusion.

A significant transient increase in MRAP at 5 minutes after administration of LPS was detected in the LS and LA groups. Comparisons of MRAP between LS and LA groups could not be performed because the groups were significantly different at –0.5 hours. The MRAP was significantly decreased at 0.5 to 2.5 hours, 5 minutes after infusion, and 5 to 5.5 hours in the LA group and at 0.5 hours to 5 minutes after infusion in the SA group.

**Metabolic variables**—Arterial pH was significantly increased in LS horses from 0 to 8 hours, except at 6 hours. A significant decrease over time for $P_{CO_2}$ was observed in the LA, LS, and SS groups. In LA horses, $P_{O_2}$ was significantly decreased from 0.5 to 2 hours. Bicarbonate concentrations and base excess were significantly decreased over time in all groups. Total $CO_2$ was significantly decreased in LA, LS, and SS groups over time. In SA horses, there was a transient decrease. The $Sa_{O_2}$ was significantly decreased in LA horses from 0.5 to 2 hours. There was a significant increase in $Ca_{O_2}$ in LA (0.5 and 2 to 6 hours) and SA (0.5 to 4 hours) horses. Oxygen delivery significantly increased in LA (1 to 2 hours) and SA (0.5 to 2 hours) horses.

**Hematologic variables**—The RBC count was significantly increased in LA (0.5, 2 to 8, and 18 hours),

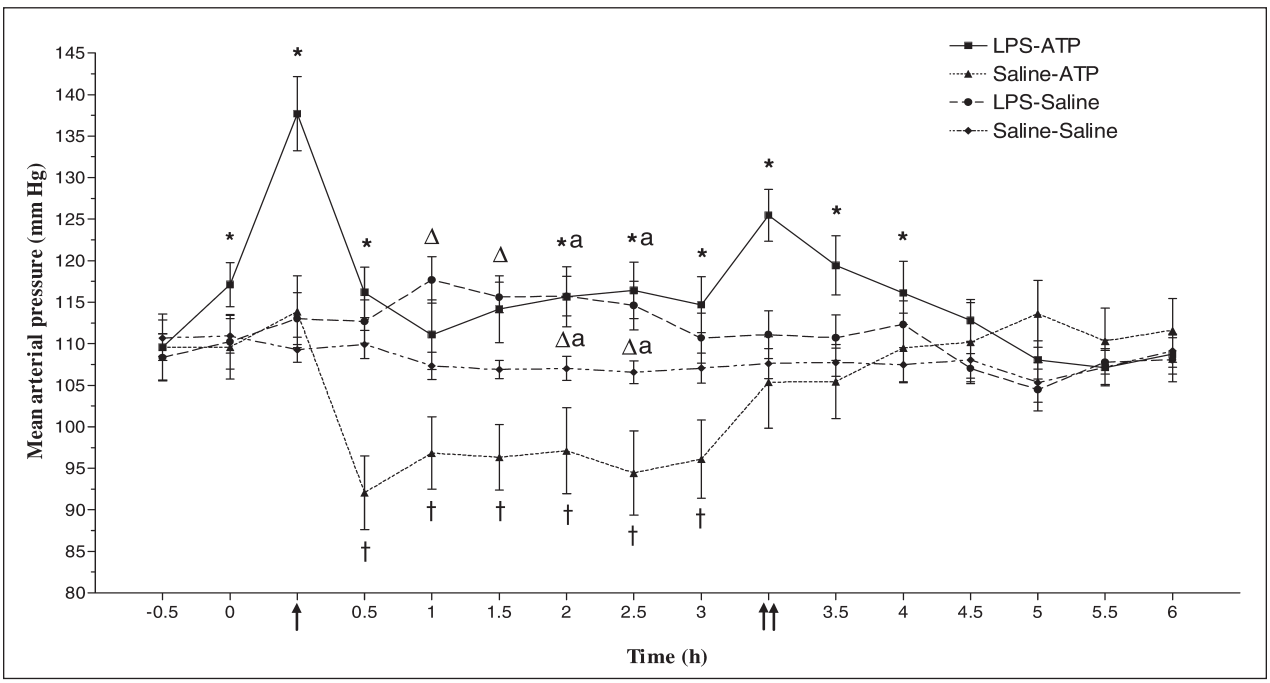



Figure 3—Mean ± SEM mean arterial pressure in horses administered a pretreatment infusion of LPS or an equivalent volume of saline solution, which was followed immediately by administration of a treatment solution of ATP-$MgCl_2$ or an equivalent volume of saline solution. *See* Figures 1 and 2 for key.

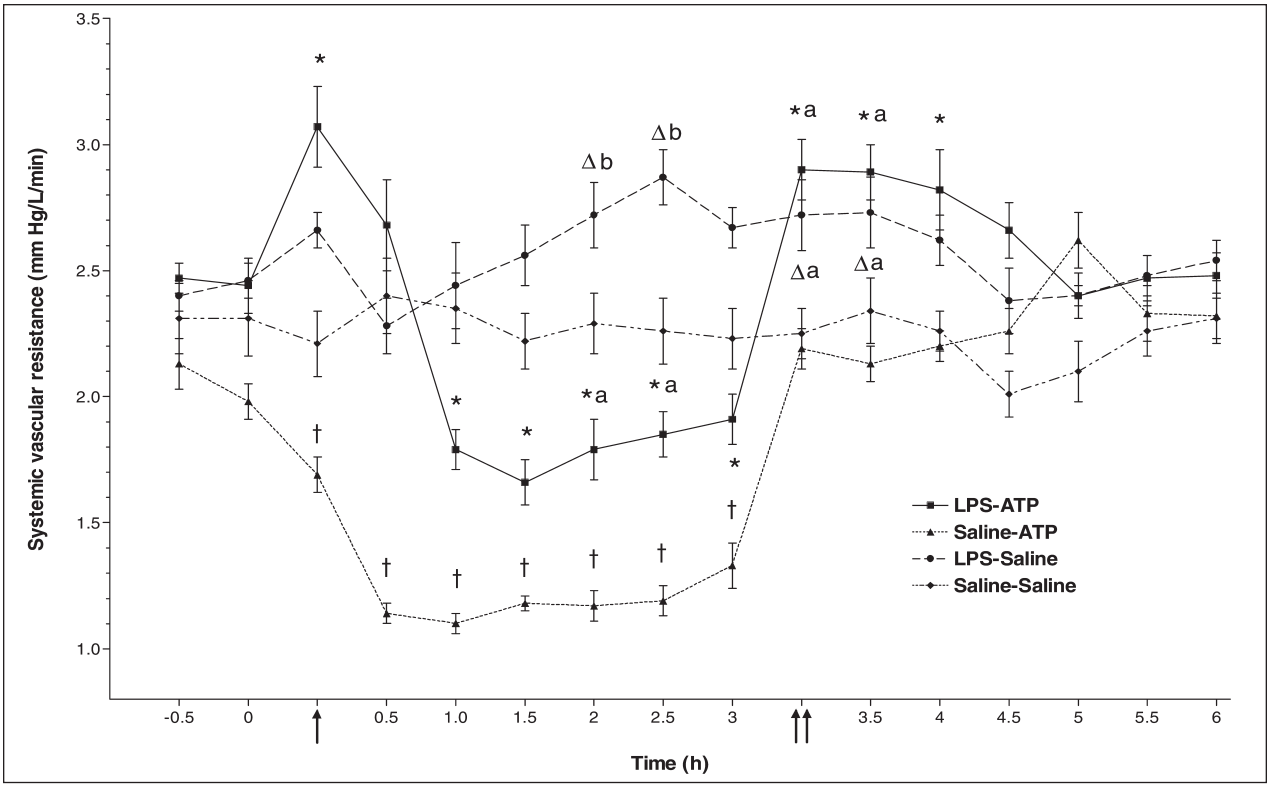



Figure 4—Mean ± SEM systemic vascular resistance in horses administered a pretreatment infusion of LPS or an equivalent volume of saline solution, which was followed immediately by administration of a treatment solution of ATP-$MgCl_2$ or an equivalent volume of saline solution. a,b—For a specific time point, between-group comparisons (LA and LS groups) were made, and values differed significantly ($P < 0.05$) between the LA and LS groups. *See* Figures 1 and 2 for remainder of key.

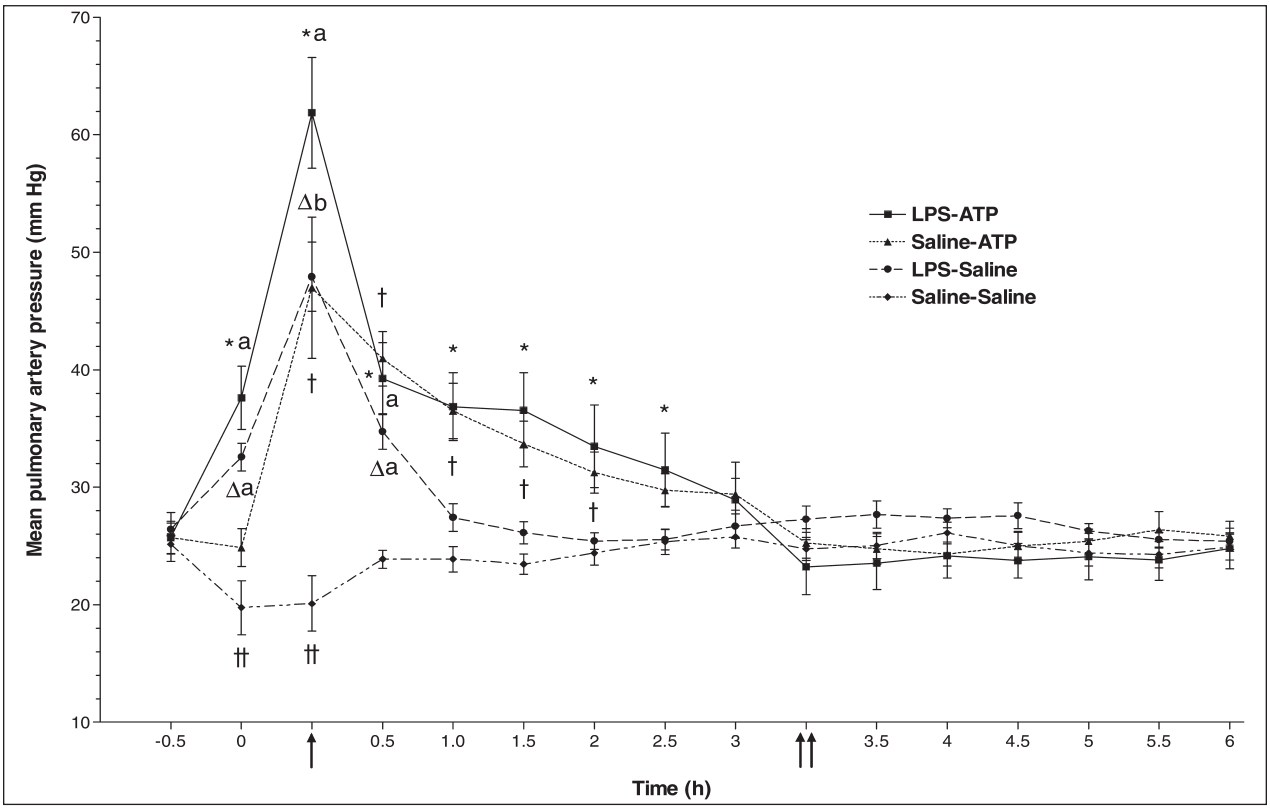



Figure 5—Mean ± SEM mean pulmonary artery pressure in horses administered a pretreatment infusion of LPS or an equivalent volume of saline solution, which was followed immediately by administration of a treatment solution of ATP-$MgCl_2$ or an equivalent volume of saline solution. ††Within the saline-saline [SS] group, value differs significantly ($P < 0.05$) from value for –0.5 hours. *See* Figures 1, 2, and 4 for remainder of key.

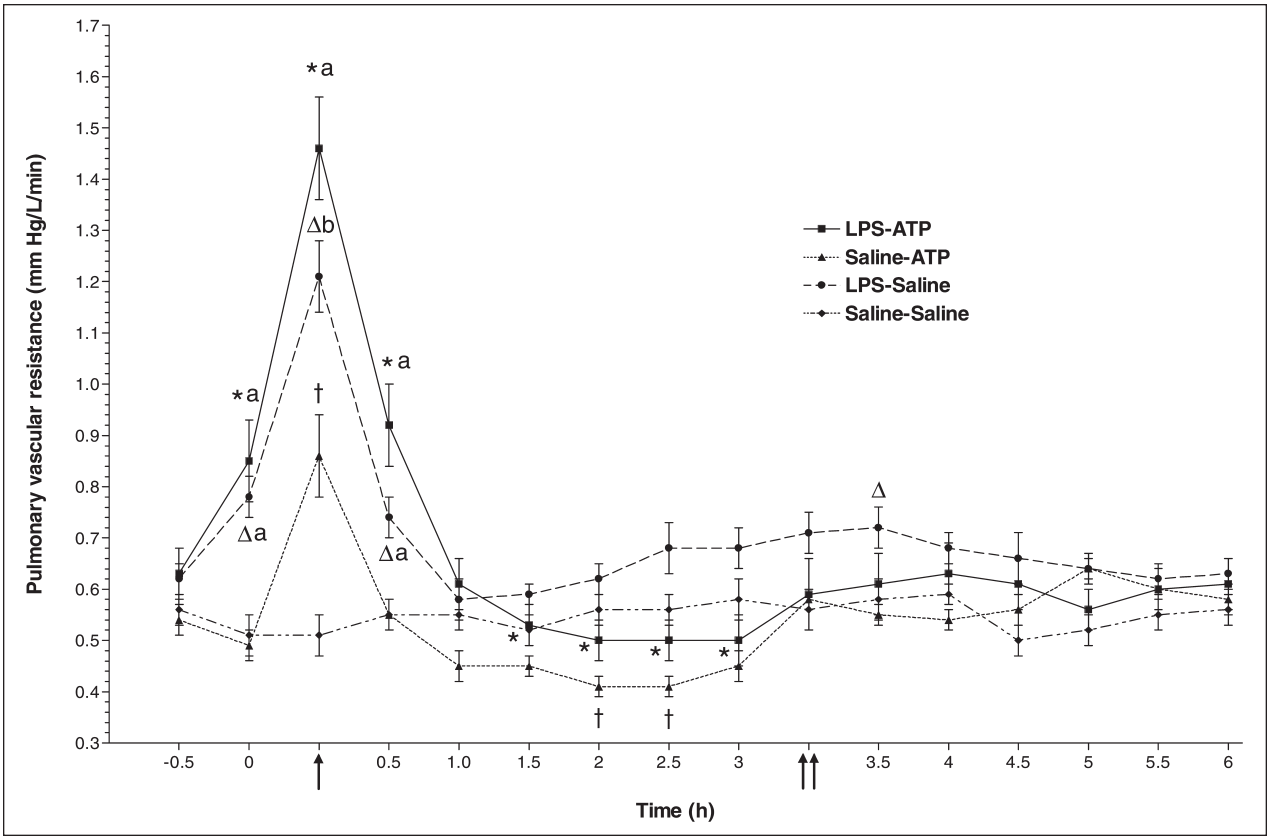
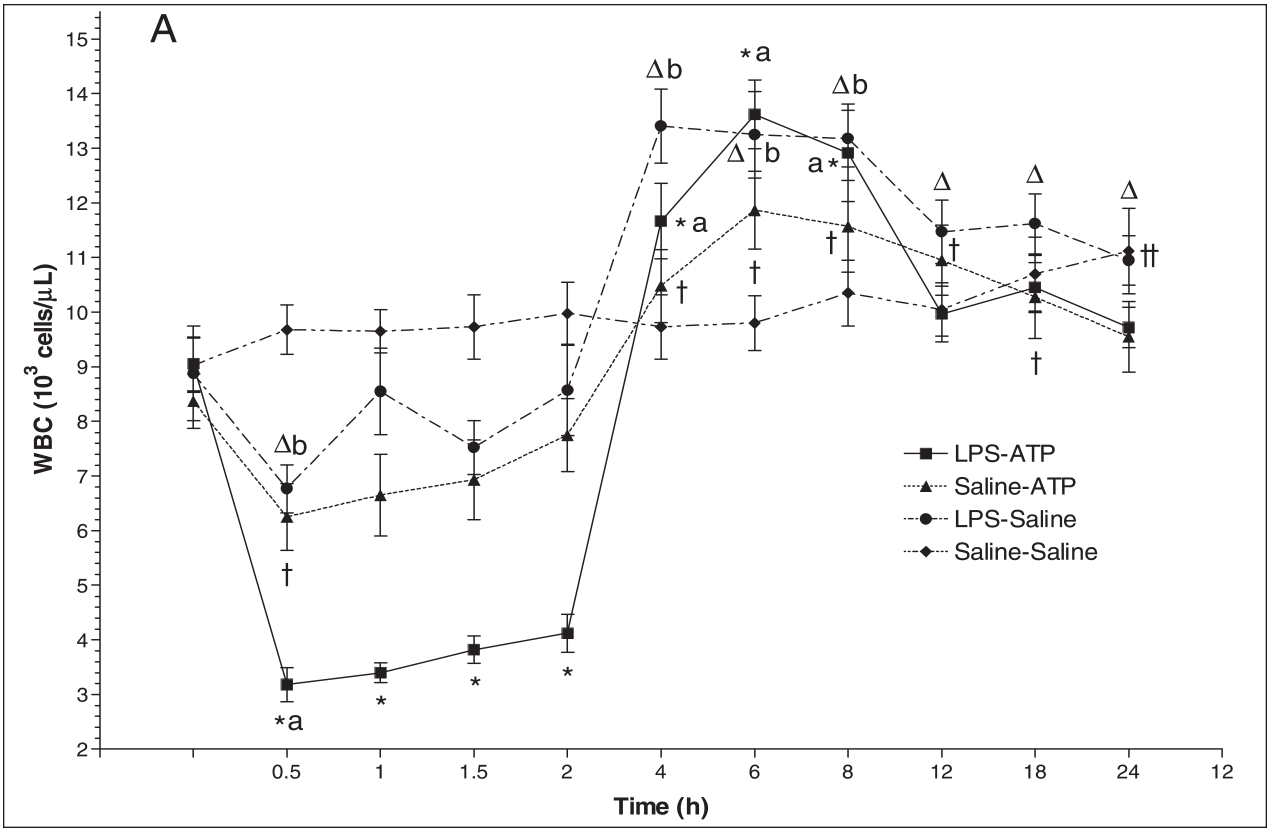



Figure 6—Mean ± SEM pulmonary vascular resistance in horses administered a pretreatment infusion of LPS or an equivalent volume of saline solution, which was followed immediately by administration of a treatment solution of ATP-$MgCl_2$ or an equivalent volume of saline solution. *See* Figures 1, 2, and 4 for key.

SA (0.5 to 4 hours), and SS (18 to 24 hours) horses. Hemoglobin concentration was significantly increased in LA (0.5, 4 to 8, and 18 hours) and SA (1 to 2 hours) horses. Number of platelets was significantly decreased in the LA group at 0.5 to 2 hours and again at 12 to 24 hours. There were significant, transient, inconsistent decreases in the number of platelets in the other 3 groups. Fibrinogen concentration was significantly decreased in LA horses at 4 hours. The WBC count decreased significantly and then increased in LA, LS, and SA horses (**Fig 7**). There was a transient significant increase in SS horses. The percentage of neutrophils was significantly decreased in LA horses from 0.5 to 2 hours and then increased in LA, LS, and SA horses beginning at 4 hours (Fig 7). Percentage of band neutrophils was significantly increased in LA horses at 4 hours. Percentage of lymphocytes was significantly decreased over time in LA, LS, and SA horses. Percentage of monocytes was significantly increased in LS (6 hours) and SS (8 and 12 hours) horses. There were no changes in the percentage of basophils over time for any group. Percentage of eosinophils was significantly decreased in LA (0.5 to 24 hours) and LS (6 hours) groups.

**Serum biochemical variables**—We did not detect consistent significant changes over time for glucose and sodium concentrations and aspartate transaminase activity in any group. In the LA group, γ-glutamyltransferase and ALP activity, total bilirubin concentration, and values for the anion gap increased significantly over time. A transient significant increase was detected in CK activity and concentrations of BUN, chloride, and magnesium. Creatinine concentrations had a transient significant increase and then a transient significant decrease. Phosphorus concentration increased significantly and then decreased over time. Potassium concentrations had a transient significant decrease and then a transient significant increase. In the SA group, ALP and CK activity; concentrations of albumin, creatinine, calcium, and magnesium; and values for the anion gap had a transient significant increase. Total protein and globulin concentrations significantly increased over time. Phosphorus concentration significantly increased and then decreased over time. In the LS group, total protein, albumin, globulin, calcium, and potassium concentrations had a transient significant increase. Creatinine and phosphorus concentrations significantly decreased over time, and chloride concentration significantly increased over time. In the SS group, total protein concentration and values for the anion gap had a transient significant increase. Albumin, calcium, potassium, chloride, and magnesium concentrations significantly increased over time. Phosphorus concentration significantly decreased over time.

**Serum cytokine activity**—The TNF-α activity did not differ significantly between SA and SS horses.



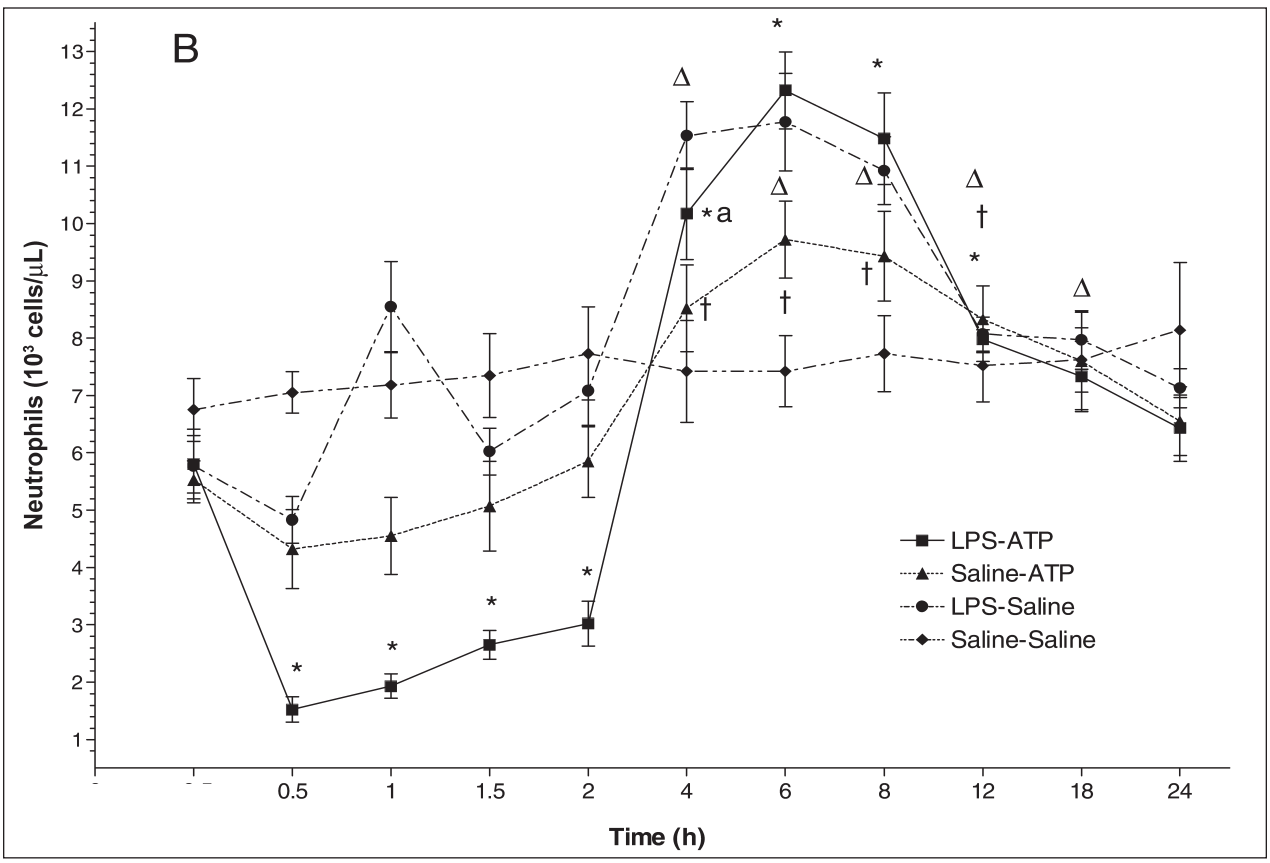




Figure 7—Mean ± SEM WBC (A) and neutrophil (B) counts in horses administered a pretreatment infusion of LPS or an equivalent volume of saline solution, which was followed immediately by administration of a treatment solution of ATP-$MgCl_2$ or an equivalent volume of saline solution. *See* Figures 1, 2, 4, and 5 for key.

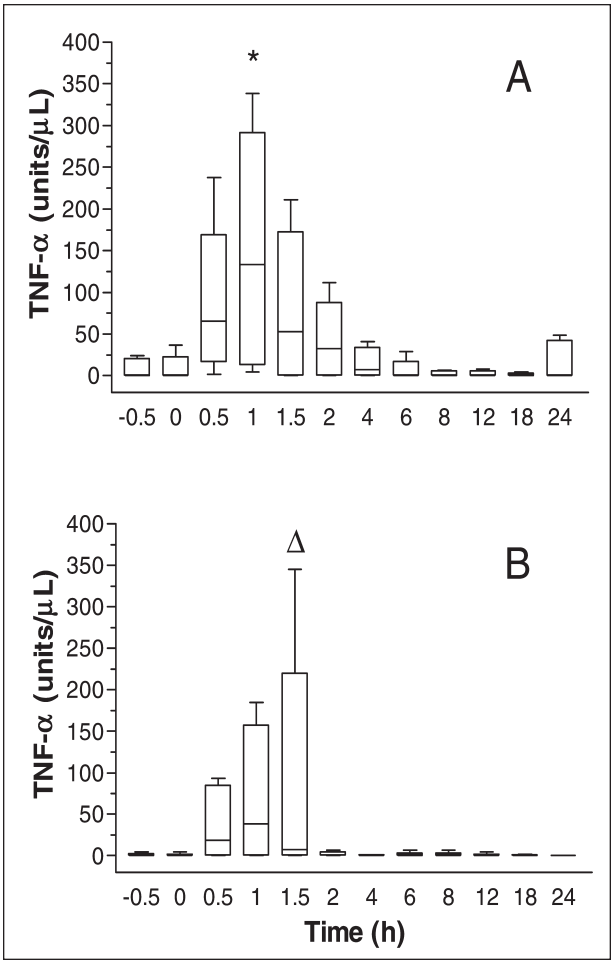



Figure 8-Box-and-whisker plots of tumor necrosis factor-α (TNF-α) in horses administered a pretreatment infusion of LPS, which was followed immediately by administration of a treatment solution of ATP-MgCl2 (LA group; A) or an equivalent volume of saline solution (LS group; B). Each box represents the central 50% of the values, the horizontal line within each box represents median values, and the whiskers represent the range of values. See Figures 1 and 2 for key.

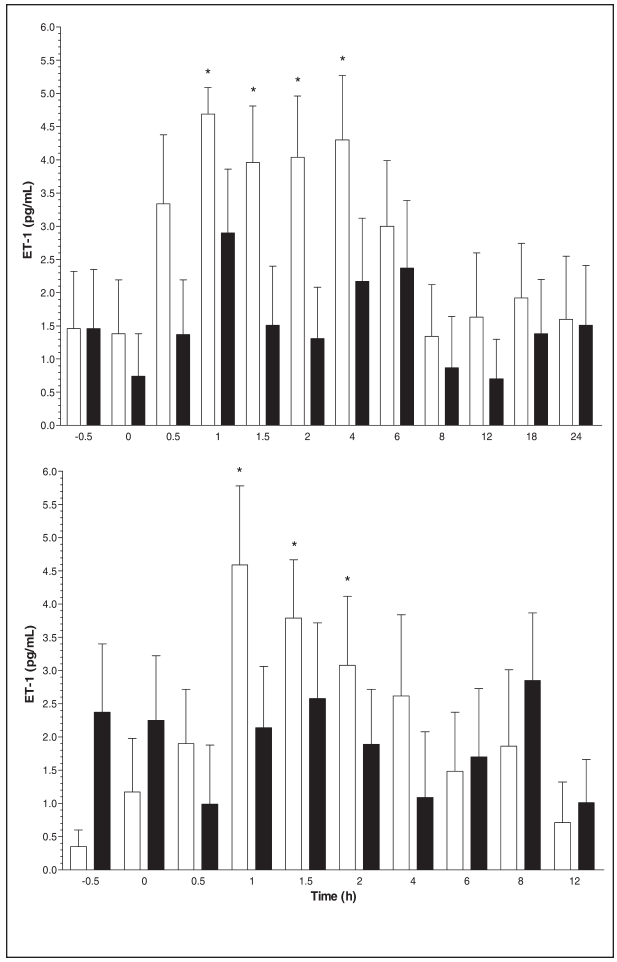



Figure 9-Mean ± SEM endothelin-1 (ET-1) concentrations in jugular venous (top) and pulmonary arterial (bottom) blood samples in horses administered a pretreatment infusion of LPS, which was followed immediately by administration of a treatment solution of ATP-MgCl2 (LA group; white bars) or an equivalent volume of saline solution (LS group; black bars). See Figure 1 for key.

However, there were significant differences in TNF-α activity over time in the LA and LS horses (**Fig 8**). The IL-6 activity did not differ over time for any group.

**ET-1 concentrations**—We did not detect significant differences in ET-1 concentrations in the jugular venous samples obtained from SA and SS horses. However, there were significant differences over time in the LA horses, and there was a significant difference in the LS horses in ET-1 concentration between the baseline sample and the sample obtained at –0.5 hours (**Fig 9**). When compared with the value at –0.5 hours, there were no significant differences among samples obtained at other time points. However, when compared with the baseline concentration, there were significant increases over time for samples obtained at other time points. There were no significant differences over time for ET-1 concentrations measured in the pulmonary artery samples collected from LS, SA, and SS horses. However, in the LA group, a significant increase was observed from 1 to 2 hours.

## Discussion

Intravenous infusion of ATP-$MgCl_2$ after administration of a low dose of endotoxin to healthy, conscious, adult horses failed to attenuate clinical, hemodynamic, metabolic, hematologic, and cytokine alterations that develop secondary to endotoxin exposure.[9,32] The combination of endotoxin and ATP-$MgCl_2$ appeared to potentiate pulmonary hypertension, leukopenia, and neutropenia that were evident when endotoxin was given alone. The combination of endotoxin and ATP-$MgCl_2$ led to thrombocytopenia. Finally, ET-like immunoreactivity was transiently increased in plasma obtained from the jugular vein and pulmonary artery in LA but not LS horses.

Pulmonary hypertension has been documented in horses given endotoxin[9,33] or ATP-$MgCl_2$.[23,24] The pulmonary hypertensive effects of ATP-$MgCl_2$ and endotoxin in the study reported here appeared to be additive. However, pulmonary hypertension and secondary hypoxemia in the LA group were transient. Furthermore, oxygen delivery was increased in this group as a result of an increase in CO. Therefore, pul-

monary hypertension and hypoxemia observed in the LA horses may not have been clinically important because oxygen delivery was increased.

Development of pulmonary hypertension secondary to ATP-$MgCl_2$ administration appears to be unique in horses. Pulmonary hypertension secondary to cardiac abnormalities in children[34] or baby pigs as a result of sepsis[35] can be successfully reversed with administration of ATP-$MgCl_2$. Attenuation of pulmonary hypertension secondary to endotoxin administration was not observed in the study reported here. The mechanism involved in ATP-$MgCl_2$-induced pulmonary hypertension in horses is not known but may be a result of differences in the density of purinergic receptors in the pulmonary vasculature. Adenosine triphosphate can cause vasoconstriction or vasodilatation, principally by activation of purinergic P2X or P2Y receptors, respectively.[36] In contrast to P2Y receptors, which are located on vascular endothelium and require generation of second messengers, P2X receptors are located on vascular smooth muscle cells and use ligand-gated ion channels to induce a response.[36] Therefore, the response time is faster with activation of P2X receptors. There may be a greater density of P2X receptors in the equine pulmonary vasculature, compared with the density in other species. Additionally, when ATP-$MgCl_2$ is administered into the external jugular vein, the pulmonary vasculature is the first major vascular bed encountered. Therefore, it will be exposed to the highest concentration of ATP. When ATP circulates through the lungs, most of it is degraded to other adenine nucleotides and nucleosides by ectonucleotidases.[37] Adenosine, a breakdown product of ATP, can activate adenosine-P1 receptors (principally $A_2$) located on vascular smooth muscle cells, which leads to vasodilatation. Vascular beds downstream from the lungs will be exposed to higher concentrations of ATP degradation products than to concentrations of ATP, thereby causing systemic vasodilatation, which was observed in the horses of our study.

Horses administered endotoxin, irrespective of subsequent treatment, developed systemic hypertension, whereas horses administered ATP-$MgCl_2$ alone developed systemic hypotension. The IV administration of ATP-$MgCl_2$ can produce a rate-dependent decrease in MAP and $SR_L$, principally via vasodilatation.[23,24] The decrease in $SR_L$ despite systemic hypertension in LA horses was secondary to an increase in CO, which was not observed in LS horses. The reduction in $SR_L$ observed in LA horses could have led to improved peripheral perfusion.

The number of WBCs decreased in horses administered endotoxin, and the magnitude of the decrease was more pronounced in the group receiving ATP-$MgCl_2$. Neutropenia was only documented in the LA group. Horses receiving ATP-$MgCl_2$ or LPS alone developed mild, transient leukopenia without corresponding neutropenia. The development of leukopenia in SA horses and the difference in the leukocyte-neutrophil response between LA and LS horses may have been attributable to upregulation of adhesion molecules by ATP. Adenine nucleotides can act directly on leukocytes to enhance expression of the CD11b-CD18 adhesion molecule.[38] Additionally, ATP can stimulate adherence of leukocytes to cultured endothelial cells via P2Y and P2U receptor-mediated events.[39]

One potential explanation for the apparent negative effects of ATP-$MgCl_2$ on pulmonary artery pressures and leukocyte-neutrophil counts in the LA group, compared with results for the LS group, may be that the response to endotoxin in the LS group was not as severe. In contrast to results of another study[9] of endotoxin administration in horses, the LS horses in the study reported here did not develop tachycardia, hypoxemia, or neutropenia. Another possible explanation would be that there was endotoxin in our ATP-$MgCl_2$ preparation. Retrospectively, when the ATP-$MgCl_2$ solution was analyzed by use of the Limulus amoebocyte lysate assay, an endotoxin dose equivalent to 1 pg/kg was detected. Although speculative, we do not believe this dose would cause alterations in clinical signs or hemodynamic and clinicopathologic variables. Pulmonary hypertension is not observed in horses administered endotoxin at dosages < 30 ng/kg.[cc] Endotoxin causes systemic hypertension, whereas ATP-$MgCl_2$ alone causes systemic hypotension. Morris et al[40] documented a negative linear correlation between TNF-$\alpha$ concentrations and WBC counts. In the study reported here, a significant increase in TNF-$\alpha$ concentration was not observed in the SA group. Finally, ATP enhances neutrophil adhesion to the endothelium, which will cause a transient leukopenia.[41] We are not aware of any studies in which this dose of endotoxin was administered to horses, so definitive conclusions regarding the contribution of endotoxin in our ATP-$MgCl_2$ solution to the overall changes observed in the LA horses cannot be made. However, based on the aforementioned information, we strongly believe that the 1 pg of endotoxin/kg in our ATP-$MgCl_2$ preparation did not significantly affect our results.

The LA but not LS horses developed thrombocytopenia during ATP-$MgCl_2$ infusion. Endotoxin can induce platelet aggregation in horses.[42] Adenosine diphosphate is an agonist of platelets and is found, along with ATP, in platelet-dense granules.[43,44] Following exposure to endotoxin, ADP is released from platelet-dense granules, contributing to platelet aggregation.[45,46] The combination of endotoxin and ATP-$MgCl_2$, some of which will be found in the circulation as ADP, most likely contributed to the LA group's development of thrombocytopenia via platelet aggregation.

Significant increases in ET concentrations above baseline values were detected at 1 to 4 hours (venous plasma) and 1 to 2 hours (pulmonary arterial plasma). These increases were detected only in horses receiving ATP-$MgCl_2$ after administration of a low dose of endotoxin (LA group). Horses receiving saline infusion after LPS did not have increases in concentrations of ET-1. In contrast to results for several other species,[47-49] the horses reported here did not have increased systemic circulating concentrations of ET-1 after administration of a low dose of endotoxin alone. However, administration of a larger dose of endotoxin may have caused increased ET-1 synthesis and release. It is also possible that there was increased ET-1 expression and local

release in some organs, but it was not detected systemically in these horses. Approximately 80% of ET-1 is released abluminally toward the smooth muscle where it binds to ET receptors to cause vasoconstriction.[50]

The early increase in MPAP in horses receiving ATP-$MgCl_2$ or saline solution after LPS was evident by the end of the LPS infusion (time 0) and continued for 30 minutes after infusion. This early pulmonary hypertension developed before there were measurable increases in ET-1 concentrations, and it was evident in horses of both groups (LA and LS). As documented in horses and other animals, this would suggest that pulmonary hypertension was likely mediated by another vasoconstrictor substance, such as thromboxane.[51-53] On the other hand, the persistent increase in MPAP from 1 to 2.5 hours in horses receiving ATP-$MgCl_2$ may have been mediated in part by increased ET-1 release in the pulmonary vasculature because the ET-1 concentration in pulmonary arterial plasma was increased from 1 to 3 hours.[53]

Although not measured in the study reported here, it is possible that the decrease in intestinal blood flow and perfusion reported[54] in animals administered LPS may be mediated in part by an increase in local synthesis and release of ET-1 in the splanchnic circulation. Horses with naturally acquired gastrointestinal tract disease have greater concentrations of circulating ET-like immunoreactivity in jugular venous plasma, compared with concentrations in healthy control horses.[55] Horses with intestinal strangulation obstruction, enterocolitis, and peritonitis had the greatest plasma concentrations of ET-like immunoreactivity in that study. Because of the type of disease and severity of mucosal injury, horses with these conditions typically are more likely to have circulating endotoxin, compared with endotoxin concentrations in other types of gastrointestinal tract disease. The difference in plasma ET concentrations between the horses in that study[55] and the horses receiving LPS in the study reported here could be that the horses with those naturally acquired diseases may have absorbed larger amounts of endotoxin.

In the study reported here, administration of ATP-$MgCl_2$ was not protective during experimentally induced endotoxemia in healthy, conscious, adult horses. Furthermore, administration of ATP-$MgCl_2$ during endotoxemia may worsen the hemodynamic, metabolic, and hematologic status of affected horses. Because ATP and other adenine nucleotides are released from cells during shock, the potential role of adenine nucleotides in the development of hemodynamic derangements, leukocyte adherence, and coagulopathies during endotoxemia warrants further investigation.

[a]Quik-Cath 2N-11-10, Baxter Healthcare Corp, Deerfield, Ill.
[b]Intramedic polyethylene tubing, model PE205, Becton-Dickinson, Sparks, Md.
[c]Pentalumen thermodilution catheter 41216-01, Abbott Critical Care Systems, Abbott Laboratories, North Chicago, Ill.
[d]Angiocath 382269, Becton Dickinson Infusion Therapy Systems Inc, Sandy, Utah.
[e]Intramedic polyethylene tubing, model PE260, Becton-Dickinson, Sparks, Md.
[f]Normosol, Abbott Laboratories, North Chicago, Ill.
[g]Injector 500, Columbus Instruments, Columbus, Ohio.
[h]Arrow radial artery catheterization set RA-04020, Arrow International Inc, Reading, Pa.
[i]DTX plus DT-6012, Becton Dickinson Infusion Therapy Systems Inc, Sandy, Utah.
[j]Cardio Max II model 85 thermodilution cardiac output computer, Columbus Instruments, Columbus, Ohio.
[k]Polygraph, model 7D, Grass Instruments, Quincy, Mass.
[l]Chart recorder, model 25-60, Grass Instruments, Quincy, Mass.
[m]Lipopolysacccharide L2880, Sigma-Aldrich Inc, St Louis, Mo.
[n]Lifecare 4, Abbott Laboratories, North Chicago, Ill.
[o]Adenosine 5′-triphosphate disodium salt A3377 and magnesium chloride hexahydrate M2670, Sigma-Aldrich Inc, St Louis, Mo.
[p]pH/blood gas analyzer, model 238, Bayer Corp, Norwood, Mass.
[q]Baker systems 9110 plus, Biochem Immunosystems Inc, Allentown, Pa.
[r]AU 600, Olympus Corp, Irving, Tex.
[s]Recombinant murine IL-6, R&D Systems, Minneapolis, Minn.
[t]96-Well flat-bottomed plates, Corning, Corning, NY.
[u]Actinomycin, Sigma-Aldrich Inc, St Louis, Mo.
[v]MTT, Sigma-Aldrich Inc, St Louis, Mo.
[w]Multiwell scanning spectrophotometer, Dynatech Laboratories Inc, Chantilly, Va.
[x]Betaplate liquid scintillation counter 1205 LKB Wallac, Turku, Finland.
[y]Aprotonin, Sigma-Aldrich Inc, St Louis, Mo.
[z]ET Kit, Biomedica, American Research Products Inc, Belmont, Mass.
[aa]Ultra microplate reader, model EL808, Bio-Tek Instruments Inc, Windsor, Vt.
[bb]SAS, version 8.0, SAS Institute Inc, Cary, NC.
[cc]Dr. Michelle Barton, Department of Large Animal Medicine, College of Veterinary Medicine, University of Georgia, Athens, Ga: Personal communication, November 2003.

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