Assessment of exercise-induced alterations in neutrophil function in horses

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Objective—To evaluate the effects of a standardized exercise test to exhaustion in horses on leukocyte function ex vivo.

Animals—6 Thoroughbred geldings.

Procedures—Blood samples were obtained from each horse before exercise; at exhaustion (termed failure); and at 2, 6, 24, 48, and 72 hours after exercise to evaluate hematologic changes, rate of leukocyte apoptosis, and leukocyte production of reactive oxygen species (ROS) ex vivo. To assess leukocyte function, leukocyte ROS production in response to stimulation with lipopolysaccharide, peptidoglycan, zymosan, and phorbol myristate acetate was evaluated. Apoptosis was evaluated via assessment of caspase activity in leukocyte lysates.

Results—In response to lipopolysaccharide, production of ROS by leukocytes was significantly increased at 2 hours and remained increased (albeit not significantly) at 6 hours after exercise, compared with the preexercise value. In the absence of any stimulus, leukocyte ROS production was significantly increased at 6 and 24 hours after exercise. In contrast, ROS production in response to phorbol myristate acetate was significantly decreased at 6, 24, and 72 hours after exercise. Leukocyte ROS production induced by zymosan or peptidoglycan was not altered by exercise. Leukocytosis was evident for 24 hours after exercise, and neutrophilia was detected during the first 6 hours. A significant increase in the rate of leukocyte apoptosis was detected at failure and 72 hours after exercise.

Conclusions and Clinical Relevance—Results indicated that strenuous exercise undertaken by horses causes alterations in innate immune system functions, some of which persist for as long as 72 hours after exercise. (*Am J Vet Res* 2007;68:1198–1204)

In horses, brief periods of strenuous exercise induce physiologic changes that are characterized by a decrease in arterial blood pH and the development of lactic acidosis.[1] Intense exercise also affects immune function. Although such effects of exercise have been studied[2] in humans, less is known about these effects of exercise in horses. In humans, it is well established that strenuous exercise impairs immune function and increases the prevalence of both subclinical and clinical infections.[2] In horses, strenuous exercise transiently impairs neutrophil antimicrobial functions and nonspecific defense mechanisms, whereas its effects on other innate and adaptive immune functions are more prolonged.[3] For example, endurance horses that were prepared for 3-day eventing had significant decreases in the in vitro killing rate of phagocytosed yeast cells during intensive exercise, compared with findings during mild exercise.[4] Similarly, in horses undergoing a standardized exercise test to 115% maximal oxygen consumption, neutrophil antimicrobial functions and nonspecific defense mechanisms were transiently impaired.[5] In addition, there is evidence that exercise-induced alterations in immune function increase the risk of infection; ponies undergoing a 5-day strenuous exercise program had decreased cell-mediated adaptive immune responses to influenza virus and increased susceptibility to infection, compared with nonexercised control ponies.[6,7]

Certain effects of exercise on immune function are identified through comparisons of leukocyte functions before and after exercise. The effects of strenuous exer-

Abbreviations

ROS	Reactive oxygen species
LPS	Lipopolysaccharide
PGN	Peptidoglycan
ZYM	Zymosan
PMA	Phorbol myristate acetate
MPO	Myeloperoxidase
AFU	Arbitrary fluorescence unit
TNF	Tumor necrosis factor
TLR	Toll-like receptor
NOD	Nucleotide-binding oligomerization domain
NADPH	Reduced form of nicotinamide adenine dinucleotide phosphate

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cise on the function of leukocytes after strenuous exercise have been evaluated in studies of humans[8] and horses.[5,9,10] The purpose of the study reported here was to evaluate the effects of a standardized exercise test to exhaustion in horses on equine leukocyte function ex vivo. Our intent was to characterize the temporal effects of strenuous exercise on leukocyte function, apoptosis or activation of circulating neutrophils, and circulating leukocyte populations. Leukocyte function was assessed by the cells' capacity to synthesize ROS in response to stimulation with LPS, PGN, ZYM, and PMA (a direct stimulator of protein kinase C). Last, apoptosis or activation of neutrophils was evaluated via assessment of caspase or MPO activity in leukocyte lysates, respectively.

Materials and Methods

Animals and treadmill incremental exercise stress testing—Six Thoroughbreds (mean age, 5 years) from the Equine Performance Laboratory at the University of Florida College of Veterinary Medicine were used in the study. The study protocol was approved by the University of Florida Institutional Animal Care and Use Committee (Permit A-130). The horses were housed in small paddocks, fed 2 kg of a commercially available concentrate twice daily, and provided hay and water ad libitum.

Prior to the start of the study, each horse underwent a period of training on a high-speed treadmill[a] for at least 8 weeks. The training program included a warm-up period involving trotting (4 m/s) for a distance of 0.6 km, followed by galloping (8 m/s) for a distance of 3.0 km and recovery at a trot (4 m/s) for a distance of 0.4 km on Monday, Wednesday, and Friday of each week.

To conduct the standardized exercise test for the study, each horse underwent a 2-minute warm-up period at 4 m/s and 0° incline, after which the speed and incline of the treadmill were increased to 8 m/s and 6°, respectively. Each horse was exercised at 8, 9, 10, 11, 12, and 13 m/s for 1 minute each until it was unable to maintain position on the treadmill because of fatigue despite humane encouragement; this point was defined as failure. At failure, the incline and speed of the treadmill were rapidly reduced, and the horse was allowed to exit the treadmill. Exercise duration and treadmill speed at failure were recorded.

Assessment of venous pH and plasma lactate concentration—For each horse, blood samples were collected from a jugular vein within 30 minutes before starting the standard exercise test; at failure; and at 2, 6, 24, 48, and 72 hours after exercise. Ten milliliters of blood was collected into a vacuum-evacuated tube[b] containing heparin (14.3 U/mL) for analysis of pH.[c] An additional 7 mL of blood was collected into a similar tube containing 15 mg of sodium fluoride and 12 mg of potassium oxalate and kept on ice until processed. The plasma was stored frozen at –80°C until assessment of plasma lactate concentration by use of a colorimetric, enzymatic method[d] that is specific for L-lactate. Lactate concentration determinations were made within 7 days after blood sample collection.

Leukocyte isolation—Sixty milliliters of blood was collected from a jugular vein of each horse into a 60-mL syringe containing 1.5 mL of sterile 100mM EDTA solution (pH, 7.2)[e] prior to the start of the standard exercise test; at failure; and at 2, 6, 24, 48, and 72 hours after exercise. Within 60 minutes after collection, the blood samples were transported to the laboratory. Each blood sample was centrifuged (1,200 × *g* for 20 minutes at 20°C) to prepare a buffy coat, which was transferred to a single 50-mL tube. Contaminating erythrocytes were removed by 2 cycles of hypotonic lysis, and the leukocytes were suspended (3×10^6 viable cells/mL) in RPMI-1640 (without phenol red[f]) containing 10% fetal bovine serum,[g] 2mM L-glutamine,[f] 1mM sodium pyruvate,[f] and 50 µg of gentamycin sulfate/mL.[e] The leukocytes were used for assessment of caspase activity (an indication of apoptosis) and the production of ROS.

Hematologic assessments—At each time point, 5 µL of blood from each horse was analyzed for total leukocyte count (by use of an automated analyzer[h]) and WBC differential. A commercially available reference standard was used to standardize data among horses.[h]

Apoptosis, which is a natural process used to remove damaged, altered, or inappropriately activated cells, can be assessed by monitoring the activity of a family of aspartate-specific cysteinyl proteases called caspases.[11] As an indicator of apoptosis, a commercial kit[i] that measures caspase 3 and caspase 7 activities simultaneously by use of a DEVD (Asp-Glu-Val-Asp) peptide that acts as a substrate for both enzymes was used. Briefly, 100 µL of caspase-3/7 reagent was added to 100 µL of unstimulated leukocytes (3×10^5 cells/well) or medium in a 96-well (flat-bottom) plate. The plates were gently agitated on a shaker, then placed in the dark at room temperature (22°C) for 2 hours. The plates were evaluated by use of a fluorescence plate reader[j] (excitation wavelength, 485 nm; emission wavelength, 538 nm) to monitor conversion of the reporter dye from the nonfluorescent to the fluorescent form. Values are reported as AFUs; specific dye conversion (reported as sp-AFUs) was calculated as the AFU value of the stimulated sample minus the AFU value of the unstimulated cells.

ROS production—Production of ROS was assessed by use of the fluorescent dye dihydrorhodamine 123,[12,k] which primarily interacts with H_2O_2. Leukocytes were placed in quadruplicate wells of a 96-well (flat-bottom) culture dish in 100 µL of either RPMI alone (unstimulated leukocytes) or in medium containing the specified treatment. Ten microliters of *Escherichia coli* O55:B5 LPS (final concentrations, 10 and 100 ng/mL),[l] ZYM (final concentrations, 100 ng/mL and 1 µg/mL),[k] PGN (final concentrations, 100 ng/mL and 1 µg/mL),[k] or PMA (final concentration, 10^{-7}M)[e] were added to quadruplicate wells. The concentrations used were based on results of previous experiments in which optimal ROS production was detected after incubation of equine leukocytes with the same stimuli (data not shown). Subsequently, 10 µL of dihydrorhodamine 123 (10µM final concentration) was added to each well, except for those wells in which medium alone was used as a medium background. The plates were incubated

for 2 hours at 37°C and then evaluated by use of a fluorescent plate reader[j] (excitation wavelength, 485 nm; emission wavelength, 538 nm) to monitor conversion of the dye from the nonfluorescent to the fluorescent form. Fluorescence data were obtained as AFU values. In each assay, PMA was used as a maximal positive stimulus and RPMI-1640 medium was used as a negative control sample. Production of ROS at each concentration of each stimulus is presented as a response ratio, calculated as follows:

$$\text{Response ratio} = \frac{\text{AFU value of stimulated cells}}{\text{AFU value of unstimulated cells}}$$

Leukocyte MPO activity—At each sample collection time, 2×10^7 leukocytes were pelleted in 1.5-mL microfuge tubes via centrifugation at $1{,}000 \times g$ for 2 minutes. The medium was aspirated, and the cell pellets were stored at –80°C until analyzed for MPO activity. The assays were performed with a fluorescent substrate MPO activity kit.[m] The pellets were thawed at room temperature, and the cell lysate was suspended in 1× PBS solution. To assess the chlorination activity of MPO, 50 µL of each sample or MPO standard was pipetted into quadruplicate wells of a 96-well microplate. To each well, 50 µL of 2× nonfluorescent 3′-(p-aminophenyl) fluorescein working solution was added. The plate was incubated at room temperature for 30 minutes in the dark, after which fluorescence was measured (excitation wavelength, 485 nm; emission wavelength, 530 nm). The data were recorded as AFU values. To standardize results for each horse, the fluorescence intensity of the zero-MPO standard was subtracted from each experimental sample and standard.

To assess peroxidation activity of MPO, 50 µL of each experimental sample or MPO standard was pipetted into quadruplicate wells of a 96-well microplate. Fifty microliters of 2× MPO kit reagent working solution was added to all wells, and the plates were incubated at room temperature for 30 minutes in the dark. The reactions were stopped by addition of 10 µL of 10× peroxidation inhibitor or 10× chlorination inhibitor to all wells. Fluorescence was measured (excitation wavelength, 530 nm; emission wavelength, 590 nm) and data were adjusted for each horse by subtracting the fluorescence intensity of the zero-MPO standard from each experimental sample and standard. Fluorescence values for the standards were plotted to provide a relative calibration of the level of MPO peroxidation enzyme activity.

Statistical analysis—Internal clustering of ROS and MPO raw fluorescent measurement data was first evaluated by use of a Q test for rejection of outliers among the 4 replicates within each individual measurement from each horse. Datum points were rejected when the Q value exceeded 0.76.[13] The Q test was used to identify and reject outliers within the raw data, and only 1 value within any set of replicates could be rejected by use of this test. Because a maximum of 1 of the 4 datum points within any raw data set could be removed, at least 75% of the replicates in each individual measurement were used for data analysis. Individual Student *t* tests were conducted to compare the means of preexercise values with the mean values for all horses at each postexercise time point (ie, at failure and at 2, 6, 24, 48, and 72 hours after exercise). The same analysis was used to evaluate effects of exercise on venous pH, plasma lactate concentration, hematologic variables (WBC, neutrophil, lymphocyte, and monocyte counts), ROS production, caspase activity, and MPO activity.[n] Significance was set at a value of $P < 0.05$.

Results

Validation of the standardized exercise test—The duration of the standardized exercise test to failure was approximately 12 minutes; the mean speed attained by the horses at this time was 11 m/s. The standardized exercise test consistently resulted in acidosis, as indicated by changes in venous pH and plasma lactate concentrations. Compared with the preexercise value, venous pH was decreased significantly at failure (mean ± SEM venous pH, 6.95 ± 0.02). The acidosis appeared to be a result of an increase in plasma lactate concentration; at failure, mean lactate concentration was 20mM. Compared with the value before the start of the exercise test, plasma lactate concentration had increased 50-fold at failure and 10-fold at 2 hours after exercise. The plasma lactate concentration returned to baseline values by 6 hours after exercise.

Hematologic variables and leukocyte apoptosis—Exercise induced changes in total leukocytes and leukocyte subpopulations (**Table 1**). Compared with preexercise findings, numbers of leukocytes and neutrophils were significantly higher after exercise. The number of circulating leukocytes was significantly greater at failure and at 2, 6, and 24 hours after exercise, compared with the value before exercise. The leukocytosis was predominately a result of neutrophilia—the numbers of neutrophils at failure and at 2 and 6 hours after the standardized exercise test were significantly increased from the preexercise value. The neutrophilia

Table 1—Mean ± SEM total and differential leukocyte counts in blood samples collected from 6 horses before strenuous exercise on a treadmill; at failure*; and at 2, 6, 24, 48, and 72 hours after treadmill exercise.

			Time after failure (h)				
Variable	**Preexercise**	**Failure**	**2**	**6**	**24**	**48**	**72**
WBCs (× 10^3 cells/µL)	8.8 ± 1.3	11.6 ± 2.6†	11.6 ± 1.5†	11.9 ± 1.9†	10.0 ± 1.1†	9.4 ± 0.7	8.6 ± 0.7
Neutrophils (× 10^3 cells/µL)	3.7 ± 1.8	6.1 ± 1.1†	6.4 ± 2.6†	5.7 ± 3.2†	4.9 ± 1.6	4.9 ± 1.3	4.6 ± 1.0
Lymphocytes (× 10^3 cells /µL)	4.2 ± 1.9	4.8 ±1.6	3.9 ± 1.7	4.5 ± 2.2	4.0 ± 1.2	3.5 ± 1.0	3.4 ± 1.0
Monocytes (× 10^3 cells/µL)	1.0 ± 0.6	0.6 ± 0.8	1.2 ± 0.8	1.3 ± 1.2	1.1 ± 0.8	1.0 ± 0.8	0.7 ± 0.7

*Failure was defined as the point at which the horse was unable to maintain position on the treadmill because of fatigue, despite humane encouragement. †Value is significantly ($P < 0.05$) different from the preexercise value of this variable.

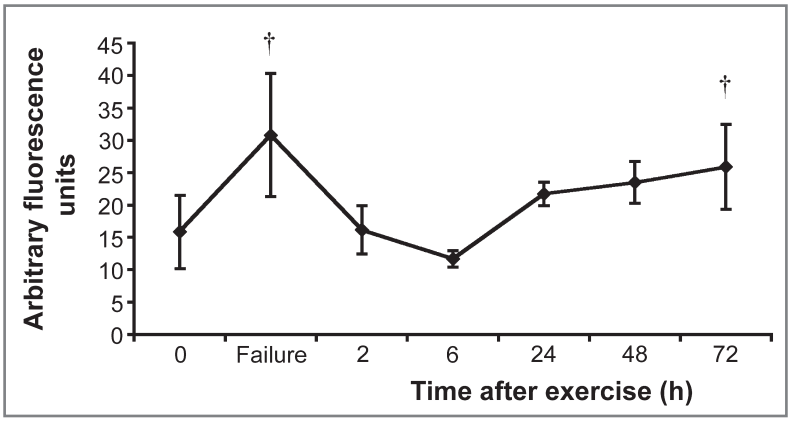

Figure 1—Alterations in apoptosis (assessed via measurement of caspase activity [expressed as AFU values]) of leukocytes in blood samples collected from 6 horses before (0 hours) strenuous exercise on a treadmill; at failure*; and at 2, 6, 24, 48, and 72 hours after treadmill exercise. Caspase activity at failure was significantly greater than the preexercise value. Values are presented as mean ± SEM. *Failure was defined as the point at which the horse was unable to maintain position on the treadmill because of fatigue, despite humane encouragement. †Value is significantly ($P < 0.05$) different from the preexercise value.

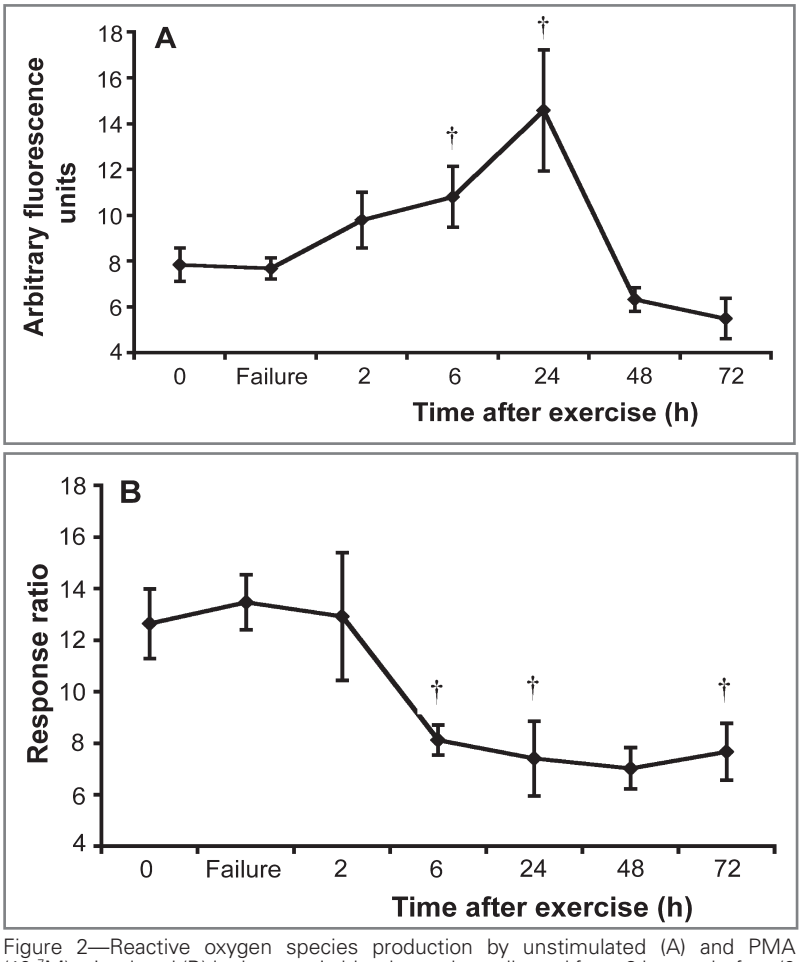

Figure 2—Reactive oxygen species production by unstimulated (A) and PMA (10^{-7}M)-stimulated (B) leukocytes in blood samples collected from 6 horses before (0 hours) strenuous exercise on a treadmill; at failure*; and at 2, 6, 24, 48, and 72 hours after treadmill exercise. Production of ROS by unstimulated cells is reported as AFU values, and PMA-induced ROS production is reported as a response ratio relative to production of ROS by unstimulated cells. Values are presented as mean ± SEM. Notice that compared with the corresponding preexercise value, ROS production by unstimulated leukocytes was significantly higher at 6 and 24 hours after exercise, whereas PMA-induced ROS production by leukocytes was significantly less at 6, 24, and 72 hours after exercise. *See* Figure 1 for key.

was resolved by 24 hours after exercise. No significant changes in lymphocytes or monocytes as a result of the standardized exercise test were detected.

The effect of exercise on apoptosis was estimated via comparison of the caspase activities of leukocytes before and after the standardized exercise test. There appeared to be a biphasic change in apoptosis as a result of exercise (**Figure 1**). Initially, apoptotic activity was increased significantly at failure, compared with the preexercise value. However, apoptotic activity subsequently decreased, and at 2 and 6 hours after exercise, values were less than that detected before exercise (albeit not significantly different). At 72 hours after exercise, apoptotic activity was again significantly increased, compared with the value before exercise.

Production of ROS—Reactive oxygen species production was monitored by use of the same number of cells in each well. As a result, the changes reported reflect the activity of a typical cell rather than a direct measure of the total activity in a horse. Compared with the preexercise value, ROS production by unstimulated leukocytes was significantly increased at 6 and 24 hours after exercise (**Figure 2**). At 48 and 72 hours, ROS production by unstimulated leukocytes had returned to values that were not significantly different from the preexercise value.

Stimulation of leukocytes with PMA was used to determine the total capacity of the neutrophils to produce ROS (**Figure 2**B). At failure and 2 hours after exercise, ROS production by leukocytes stimulated with PMA was not significantly different from the preexercise value. However, at 6, 24, and 72 hours, PMA-induced ROS production by leukocytes was significantly less than the preexercise value.

Differential patterns of ROS production were evident following incubation of leukocytes with the microbial components, LPS, PGN, and ZYM. Among the 3 ligands, leukocyte production of ROS after exercise was significantly increased (compared with the preexercise value) only when the cells were incubated with LPS at a concentration of 100 ng/mL (**Figure 3**). Production of ROS by leukocytes incubated with this concentration of LPS peaked at 2 hours after exercise; ROS production remained increased at 6 hours after exercise, although that value was not significantly different from the value before exercise. In contrast, production of ROS by leukocytes incubated with either ZYM or PGN was not significantly differ-

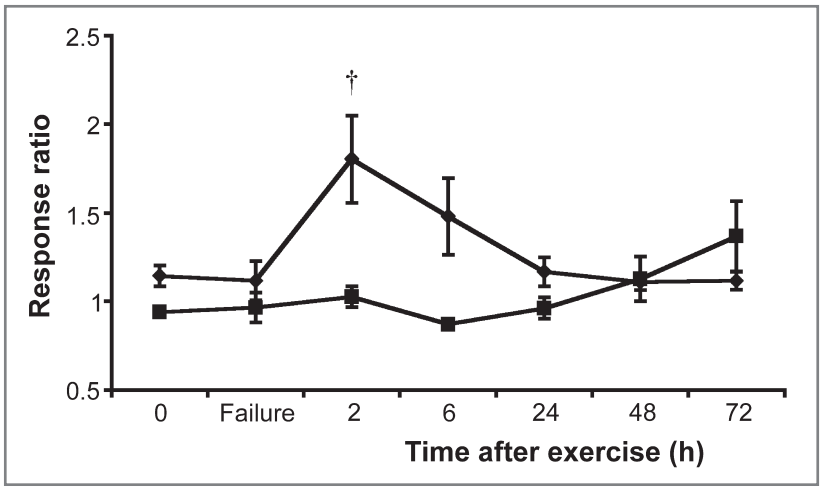

Figure 3—Production of ROS in leukocytes in blood samples collected from 6 horses before (0 hours) strenuous exercise on a treadmill; at failure*; and at 2, 6, 24, 48, and 72 hours after treadmill exercise after incubation of the cells with LPS at concentration of 100 (diamonds) or 10 ng/mL (squares). Values are presented as mean ± SEM. The LPS-induced ROS production was significantly greater than the preexercise value at 2 hours after exercise. *See* Figure 1 for key.

ent at failure or any time point after exercise, compared with the values prior to exercise (data not shown).

Neutrophil MPO activity—Relative to the preexercise value, MPO activity in leukocyte lysates did not change significantly at failure or at any point after exercise. Because all of the leukocyte pellets used in this assay contained the same number of cells (ie, 2×10^7 cells), these assessments reflected the MPO activity of a typical leukocyte rather than the total activity of the cells in circulation.

Discussion

The findings of the present study support the concept that strenuous exercise induces alterations in innate immune function in horses, some of which persist for at least 72 hours. The most striking effect of the standardized exercise test used in the study was the difference in the production of ROS by leukocytes induced by incubation with each of 3 microbial ligands. Whereas production of ROS by leukocytes incubated with LPS was significantly increased, compared with the preexercise value, at 2 hours after exercise and remained increased (albeit not significantly) at 6 hours after exercise, there was no significant effect of exercise on production of ROS induced by either PGN or ZYM.

Fetal bovine serum was included in the media in all experiments for 2 reasons. First, serum provides LPS-binding protein, an important component involved in the complex interactions between LPS and leukocytes. Second, the results of assessments of neutrophil function in culture in presence of serum are more consistent than those achieved in the absence of serum.[14] Furthermore, serum is necessary for optimal production of ROS by equine leukocytes.[15]

The selective increase in LPS-induced production of ROS in the present study may be the result of the transient endotoxemia, similar to that detected in horses after a race[16] and after completion of an endurance competition.[17] By use of the same standardized exercise test as that used in the present study, we previously determined that circulating concentrations of LPS, TNF-α, and prostaglandin $F_{2\alpha}$ were increased significantly at failure, compared with preexercise values.[18] Because LPS-mediated effects on neutrophils appear to be mediated by TNF-α and prostaglandins,[19] strenuous exercise exposes neutrophils in vivo to all 3 of these substances. Thus, the in vivo exposure to LPS, TNF-α, and prostaglandins may account for the significant enhancement in ROS production by leukocytes (both unstimulated and LPS stimulated) that were isolated from horses after the standardized exercise test in the present study.

Our finding that ROS production after exercise was not increased in response to either PGN or ZYM may be attributable to the fact that these microbial ligands interact with receptors that are distinct from the receptors that recognize LPS. Lipopolysaccharide is recognized by TLR4,[20] PGN is recognized by TLR2[21,22] and TLR6, and the muramyl dipeptide metabolite of PGN is recognized by NOD1 and NOD2.[23] The β-glucan cell component of ZYM is recognized by dectin-1, a C-type lectin pattern recognition receptor[24] that is involved in innate immune responses to fungi.[25] In the present study, production of ROS by leukocytes incubated with ZYM was decreased (although not significantly) after the exercise test, compared with the preexercise value. This finding may be a result of endotoxemia-associated downregulation of the dectin-1 receptor, a response that has been reported[24] previously for mouse leukocytes incubated with LPS.

In contrast to the increase in ROS production induced by LPS, PMA-induced ROS production by leukocytes that were isolated 6, 24, and 72 hours after completion of the exercise test was significantly decreased, compared with the value before exercise. Phorbol myristate acetate stimulates the production of ROS via stimulation of NADPH oxidase in cells, and its actions are mediated by protein kinase C activation.[26] The reduction in PMA-induced ROS production detected after exercise is indicative of a reduction in the enzymatic activity of NADPH associated with ROS production in leukocytes, which may be important during bacterial infections. Within neutrophils, NADPH oxidase–derived oxidant stress is critical for the generation of hypochlorous acid by MPO[27]; a decrease in the capacity of phagocytic leukocytes to produce ROS may be detrimental to the host.

In the present study, an indication of increased apoptotic activity in leukocytes obtained from horses at failure was detected via evaluation of the caspase 3 and 7 activities of isolated leukocytes ex vivo. These findings are consistent with those of a recent study[28] in which the apoptotic index of lymphocytes increased during exercise (compared with preexercise values) and was correlated with increasing intensity of exer-

cise. Because TNF-α is a potent inducer of apoptosis through activation of several pathways that result in programmed cell death regulated by caspase enzymes,[29] increases in circulating concentrations of TNF-α may be responsible for the increased apoptotic activity in leukocytes collected from horses at failure in the present study.

In the horses of the present study, the number of circulating leukocytes was significantly increased at failure and at 2, 6, and 24 hours after exercise, compared with the preexercise value. This increase in total leukocytes was associated with an increase in the number of neutrophils in circulation. Because the assessments of ROS production were performed in representative samples from the total leukocyte pool, it is feasible that some of the increase in ROS production detected after the exercise test may have been attributable to the increased neutrophil fraction of the cells in the test wells. However, the percentage of neutrophils in the total leukocyte population increased by only 6% at 6 hours after exercise, whereas ROS production by unstimulated leukocytes at the same sample collection time increased more than 35%. Differences between the circulating number of neutrophils and leukocyte ROS production were even more obvious at the 24-hour time point. At that time, the percentage of neutrophils in the total leukocyte population had increased 7% from the preexercise value, whereas ROS production by unstimulated leukocytes increased more than 80%. These findings suggest that most of the increase in ROS production detected in the present study was a result of changes in cellular activation status and not a result of an alteration in the composition of the leukocyte population.

The findings of the present study have suggested that strenuous exercise undertaken by horses causes alterations in the innate immune function that are evident immediately and that may persist for as long as 72 hours. In the horses of our study, strenuous exercise resulted in neutrophilia, increased rates of leukocyte apoptotic activity, and increased ROS production by unstimulated leukocytes. Furthermore, ROS production by leukocytes was increased after exercise by incubation of those cells with LPS, but not with PGN or ZYM; however, the cells' total capacity for ROS production was decreased. Additional studies are needed to determine whether the immune system changes detected in horses after exercise are detrimental or beneficial to the host.

a. High-speed treadmill (modified Sato), Equine Dynamics Inc, Lexington, Ky.
b. Vacutainer, Becton-Dickinson, Franklin Lakes, NJ.
c. Blood gas analyzer GEM Premier 3000, Instrumentation Laboratory, Lexington, Mass.
d. Trinity Biotech, Winklow, Ireland.
e. Sigma, St Louis, Mo.
f. Mediatech, Herndon, Va.
g. Fetal bovine serum, Hyclone, Logan, Utah.
h. HemaVet Inc, Oxford, Conn.
i. ApoONE, Promega, Madison, Wis.
j. Thermo Labsystems Inc, Global Medical Instrumentation Inc, Albertville, Minn.
k. Molecular Probes, Eugene, Ore.
l. List Biological Inc, Campbell, Calif.
m. EnzChek myeloperioxidase activity assay kit, Molecular Probes, Eugene, Ore.
n. Graph Pad Software Inc, San Diego, Calif.

References

1. Parks CM, Manohar M. Blood-gas tensions and acid-base status in ponies during treadmill exercise. *Am J Vet Res* 1984;45:15–19.
2. Nieman DC. Current perspectives on exercise immunology. *Curr Sports Med Rep* 2003;2:539–542.
3. Wong CW, Smith SE, Thong YH, et al. Effects of exercise stress on various immune functions in horses. *Am J Vet Res* 1992;53:1414–1417.
4. Buschmann H, Baumann M. Alterations of cellular immune response during intensive training of event horses. *Zentralbl Veterinarmed [B]* 1991;38:90–94.
5. Raidal SL, Love DN, Bailey GD, et al. Effect of single bouts of moderate and high intensity exercise and training on equine peripheral blood neutrophil function. *Res Vet Sci* 2000;68:141–146.
6. Folsom RW, Littlefield-Chabaud MA, French DD, et al. Exercise alters the immune response to equine influenza virus and increases susceptibility to infection. *Equine Vet J* 2001;33:664–669.
7. Horohov DW, Dimock A, Guirnalda P, et al. Effect of exercise on the immune response of young and old horses. *Am J Vet Res* 1999;60:643–647.
8. Peters EM, Van Eden M, Tyler N, et al. Prolonged exercise does not cause lymphocyte DNA damage or increased apoptosis in well-trained endurance athletes. *Eur J Appl Physiol* 2006;98:124–131.
9. Raidal SL, Love DN, Bailey GD, et al. The effect of high intensity exercise on the functional capacity of equine pulmonary alveolar macrophages and BAL-derived lymphocytes. *Res Vet Sci* 2000;68:249–253.
10. Raidal SL, Love DN, Bailey GD, et al. Effect of a single bout of high intensity exercise on lower respiratory tract contamination in the horse. *Aust Vet J* 1997;75:293–295.
11. Kothakota S, Azuma T, Reinhard C, et al. Caspase-3-generated fragment of gelosin: effector of morphological change in apoptosis. *Science* 1997;278:294–298.
12. Hurley DJ, Parks RJ, Reber AJ, et al. Dynamic changes in circulating leukocytes during the induction of equine laminitis with black walnut extract. *Vet Immunol Immunopathol* 2006;110:195–206.
13. Skoog DA, West DM. Rejection of data. In: *Fundamentals of analytical chemistry*. 2nd ed. New York, NY: Holt, Rinehart, and Winston Inc, 1969;45.
14. Goihman-Yahr M, Villalba-Pimentel L, Rodriguez-Ochoa G, et al. Studies on the effect of serum and proteins on in vitro-induced neutrophil activation. *J Reticuloendothel Soc* 1978;23:435–446.
15. Okano S, Hurley DJ, Bergh MS, et al. Optimization of condition for measurement of tissue factor expression and radical oxygen species induction by canine mononuclear cell and granulocytes in vitro. *Vet Immunol Immunopathol* 2006;112:234–242.
16. Baker B, Gaffin SL, Wells M, et al. Endotoxaemia in racehorses following exertion. *J S Afr Vet Assoc* 1988;59:63–66.
17. Barton MH, Williamson L, Jacks S, et al. Effects on plasma endotoxin and eicosanoid concentrations and serum cytokine activities in horses competing in a 48-, 83-, or 159-km endurance ride under similar terrain and weather conditions. *Am J Vet Res* 2003;64:754–761.
18. Donovan DC, Jackson CA, Colahan PT, et al. Exercise-induced alterations in pro-inflammatory cytokines and prostaglandin $F_{2\alpha}$. *Vet Immunol Immunopathol* 2007;118:263–269.
19. Weiss DJ, Evanson OA. Evaluation of lipopolysacharide-induced activation of equine neutrophils. *Am J Vet Res* 2002;63:811–815.
20. Shimazu R, Akashi S, Ogata H, et al. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J Exp Med* 1999;189:1777–1782.
21. Akira S. TLR Signaling. *Curr Top Microbiol Immunol* 2006;311:1–16.

22. Takeuchi O, Hoshino K, Kawai T, et al. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* 1999;11:443–451.
23. Windheim M, Lang C, Peggie M, et al. Molecular mechanisms involved in the regulation of cytokine production by muramyl peptide. *Biochem J* 2007;404:179–190.
24. Brown GD, Taylor PR, Reid DM, et al. Dectin-1 is a major beta-glucan receptor on macrophages. *J Exp Med* 2002;196:407–412.
25. Willment JA, Lin HH, Reid DM, et al. Dectin-1 expression and function are enhanced on alternatively activated and GM-CSF-treated macrophages and are negatively regulated by IL-10, dexamethasone, and lipopolysaccharide. *J Immunol* 2003;171:4569–4573.
26. Hancock JT, Desikan R, Neill SJ. Role of reactive oxygen species in cell signaling pathways. *Biochem Soc Trans* 2001;29:345–350.
27. Gujral JS, Hinson JA, Farhood A, et al. NADPH oxidase-derived oxidative stress is critical for neutrophil cytotoxicity during endotoxemia. *Am J Physiol Gastrointest Liver Physiol* 2004;287: G243–G252.
28. Navalta JW, Sedlock DA, Park K-S. Effect of exercise intensity on exercise-induced lymphocyte apoptosis. *Int J Sports Med* 2007;28:539–542.
29. Rath PC, Aggarwai BB. TNF-induced signaling in apoptosis. *J Clin Immunol* 1999;19:350–364.