Effects of extracellular lactate on production of reactive oxygen species by equine polymorphonuclear leukocytes in vitro

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Objective—To evaluate effects of extracellular lactate on viability, shape change, lactate metabolism, and reactive oxygen species (ROS) production in equine polymorphonuclear leukocytes (PMNs).

Sample—PMNs isolated from equine venous blood samples.

Procedures—PMNs were incubated with 0 to 300 mM lactate for 30 minutes before each experiment. Viability was assessed via trypan blue exclusion. Shape change was assessed via flow cytometry and light microscopy. Relative quantification of monocarboxylic acid transporter and lactate dehydrogenase lactate dehydrogenase (LDH) isotype mRNAs was performed with a real-time PCR assay. Effects of lactate at a pH of 7.4 to 6.0 on ROS production in response to phorbol 12-myristate 13-acetate, opsonized zymosan, or N-formyl-methionyl-leucyl-phenylalanine were assessed by luminol-dependent chemiluminescence.

Results—Lactate had no effect on viability of PMNs but did alter their size and density. Monocarboxylic acid transporter 1 and lactate dehydrogenase B mRNA values were not altered. Monocarboxylic acid transporter 4 and lactate dehydrogenase A mRNA values were significantly decreased. Lactate incubation of cells significantly decreased PMN-derived luminol-dependent chemiluminescence and induced different sensitivities to stimulants (phorbol 12-myristate 13-acetate, opsonized zymosan, and N-formyl-methionyl-leucyl-phenylalanine). The response ratio to N-formyl-methionyl-leucyl-phenylalanine revealed that PMNs were primed by incubation with up to 50 mM lactate, significantly increasing the production of ROS. Incubation with lactate and acidic pH caused a synergistic effect on ROS production.

Conclusions and Clinical Relevance—Extracellular lactate potentially has a direct effect on the capacity to produce ROS by equine PMNs, which may be associated with alterations in innate immune functions within a short period after high-intensity exercise. (Am J Vet Res 2012;73:1290–1298)

Lactate, which is the end product of nonoxidative glycolysis, is an important metabolite as a fuel source and gluconeogenic precursor. In addition to serving as a substrate, extracellular lactate has been implicated in various processes in humans and rodents. Extracellular lactate affects insulin resistance, cell viability, signaling molecules for the expression of various genes, innate immune activation, and the scavenging of ROS. Although lactate concentration is an important measure to determine anaerobic capacity of equine athletes, particularly those that perform at an elite level, little is known regarding the relationship between the effects and concentrations of extracellular lactate in horses.

Blood and muscle lactate concentrations increase substantially in horses after high-intensity exercise. In dynamic skeletal muscle during high-intensity exercise, lactic acid is abundantly produced and immediately dissociates into lactate anions and protons, causing a decrease of intracellular and extracellular pH. After high-intensity exercise, lactate concentrations in horses

Abbreviations

- CT: Cycle threshold
- FMLF: N-formyl-methionyl-leucyl-phenylalanine
- HBSS: Hanks balanced salt solution
- LDCL: Luminol-dependent chemiluminescence
- LDH: Lactate dehydrogenase
- LPS: Lipopolysaccharide
- MCT: Monocarboxylic acid transporter
- OZ: Opsonized zymosan
- PMA: Phorbol 12-myristate 13-acetate
- PMN: Polymorphonuclear leukocyte
- ROS: Reactive oxygen species

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are increased up to 30 mM/L or more in blood and up to 200 mmol/kg of dry weight in muscle, accompanied by pH decreases to 7.1 and 6.4, respectively. These lactate concentrations in horses are much higher than those in humans after high-intensity exercise (approx 15 mmol/L in blood and 100 mmol/kg of dry weight in muscle). The half-life of lactate in the blood is much longer in horses (≥ 30 minutes) than in humans (15 to 21 minutes). Thus, the exercise-induced lactate probably acts as a more important factor that affects physiologic functions in horses than in humans. Particularly, the substantial and prolonged increase in lactate concentrations in horses causes stress to leukocytes because lactate concentrations are related to increased osmolarity and decreased pH in blood.

Exercise alters innate immune functions in humans and rodents, causing the alteration of ROS production by PMNs, which is associated with inflammatory responses to bacterial infection and muscle injury after exercise. In horses, ROS production by equine PMNs is substantially impaired within a short period after high-intensity exercise. Furthermore, strenuous exercise reportedly induces different responses of equine leukocytes to each stimulant for ROS production. Although it is clear that exercise increases lactate concentrations in horses, it remains unknown whether the increased extra-cellular lactate concentrations associated with strenuous exercise are directly involved in alteration of equine PMN functions. However, there are some reports that extracellular lactate affects ROS production and phagocytosis of human PMNs and macrophages. Similarly, the synergistic effect of extracellular lactate and pH on ROS production by equine PMNs is not as clear as reported in human PMNs. On the basis of these findings, the possibility that extracellular lactate contributes to the alteration of equine PMN functions after exercise should be considered. The purpose of the study reported here was to elucidate the potential effect of extracellular lactate on viability, shape change, lactate metabolism, and ROS production in equine PMNs.

**Materials and Methods**

**Buffer and reagent preparation**—Hanks balanced salt solution was adjusted to a pH of 7.4, 7.0, 6.5, or 6.0 with 7.5% (wt/vol) NaHCO₃. Sodium 1-lactate was adjusted to 2M at a pH of 7.4 as a stock solution with 7.5% NaHCO₃. Sodium lactate was adjusted to a lower pH of 7.0, 6.5, or 6.0 with 2M l-(+)-lactic acid. Working solutions were made as needed at twice the concentrations of 0, 15, 30, 50, 100, 200, or 300 mM of sodium lactate with HBSS at each pH. The dilution of the solutions did not alter their pH. Phorbol 12-myristate 13-acetate and fMLF were dissolved in dimethyl sulfoxide at a concentration of 5 and 10 mM, respectively. Lipopolysaccharide serotype O55c was dissolved in HBSS at a concentration of 5 and 10 μM, respectively. Lipopolysaccharide serotype O55c was dissolved in HBSS at a concentration of 5 and 10 μM, respectively.

**Sample populations**—Blood samples were collected from 13 healthy Thoroughbreds (11 geldings and 2 stallions; mean age, 10.8 years) at rest at least 1 hour after daily maintenance exercise (walking for 30 minutes followed by trotting and cantering each for 15 minutes). The horses were owned and managed by the Nihon University Equestrian Team. Use of these horses was approved by the Animal Experiment Committee of the College of Bioresource Sciences, Nihon University.

**Equine PMN preparation and assessment of viability**—Blood samples (40 to 120 mL) were collected from the jugular vein using heparinized evacuated tubes. Erythrocytes were allowed to sediment under gravity for 30 minutes at room temperature (approx 22°C). The upper leukocyte-rich plasma layer was aspirated, collected in 15-mL polystyrene tubes, and centrifuged at 200 × g for 5 minutes. The supernatant was centrifuged at 2,000 × g for 20 minutes to prepare the platelet-poor plasma. The leukocyte-rich pellet was resuspended in 2 mL of the platelet-poor plasma and layered onto a solution of polysaccharide and sodium diatrizoate. The layered solution was centrifuged at 400 × g for 15 minutes at room temperature. After the contaminating erythrocytes in the PMN pellet were lysed by lysis buffer (150mM NH₄Cl, 14mM NaHCO₃, and 0.1mM EDTA-Na; pH, 7.3), PMNs were washed 3 times with HBSS at a pH of 7.4 (during centrifugation at 200 × g for 10 minutes at room temperature) and then suspended at a concentration of 2 × 10⁶ cells/mL in HBSS at a different pH for each experiment. The mean ± SD purity of isolated PMNs was 97.1 ± 1.8% (89.1 ± 0.5% neutrophils and 7.3 ± 5.7% eosinophils), as determined by Romanowsky staining of cytocentrifuged preparations. The viability of PMNs from 3 horses was examined immediately after isolation and incubation with 0, 30, 100, or 300 mM lactate for 30 minutes at 37°C by trypan blue exclusion and propidium iodide staining (final concentration, 1 μg/mL).

**Assessment of shape change in equine PMNs**—Changes in the shape of PMNs immediately after incubation with 0, 30, 100 or 300 mM lactate at 37°C for 30 minutes were assessed by flow cytometry and light microscopy. In flow cytometry, a population of PMNs (1 × 10⁶ cells/mL) for assessment of shape changes was determined by use of the autotagging tool from analysis software, following the validation of equine PMN viability by propidium iodide staining. The size and density of PMN populations were evaluated by forward scatter and side scatter, respectively. In the microscopic assay, cytocentrifuged PMNs were stained with May–Grunwald–Giemsa solution.

**Relative quantification of gene expression via real-time PCR assay**—Polymerase nuclear leukocytes (1.5 × 10⁶ cells) were incubated with 0, 30, 100, or 200 mM lactate for 30 minutes at 37°C, and the total RNA samples were then isolated by acid guanidinium thiocyanate-phenol-chloroform extraction. Total RNA samples were treated with DNase I to eliminate contaminating genomic DNA and then normalized to 50 ng/μL with nuclease-free water. The cDNA samples
were synthesized from 450 ng of the treated RNA in a total volume of 20 μL by use of a reverse transcription kit. The relative quantification of MCT1, MCT4, LDHA, and LDHB mRNAs was performed via real-time PCR assay and analyzed by the 2−ΔΔCT method. Primers specific for the equine MCT1 and MCT4 genes were designed with an online program (Appendix), and the β2M gene was selected as the reference gene. To perform the 2−ΔΔCT method, the equal amplification efficiency between each target and the β2M gene, which was defined as the absolute slope value of < 0.1 in the plot of the log cDNA dilution versus ΔCt, was validated as described. The specific primers for the 2−ΔΔCT method with LDHA and LDHB as the target genes were validated with the β2M gene as the reference gene in a study by our laboratory group. Real-time PCR assay was performed in duplicate for each sample with the sequence detection system. The 25-μL total reaction volume contained 2 μL of diluted cDNA (1:6), 12.5 μL of the reaction mixture, and 400nM each of the forward and reverse primers. The amplification conditions were 2 minutes at 50°C and then 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. The relative expression levels of each target gene were represented by R = 2−ΔΔCT, in which ΔΔCT = ΔCt for each concentration of lactate − ΔCt for 0mM lactate.

Chemiluminescent measurement of ROS—Luminol-dependent chemiluminescence was measured with a luminescence microplate reader that detects emission wavelengths in the range of 380 to 630 nm with peak emission near 495 nm in luminol. The LDCL represented apparent values of the ROS produced by equine PMNs, in which the effect of extracellular lactate in scavenging ROS is not considered. Background values were equivalent with or without lactate, indicating that there was no substantial effect of lactate on LDCL. The LDCL for each sample was measured in a total volume of 200 μL and in triplicate or quadruplicate wells on white 96-well flat-bottom plates for 30 minutes at 37°C. Equine PMNs (1 × 10^6 cells) were incubated in the absence (control) or presence of lactate (15 to 300mM) for 30 minutes at 37°C in a volume of 160 μL. Then, to assess unstimulated LDCL, 40 μL of luminol (final concentration, 1mM) was added to each well. In the assessment of stimulated LDCL, 40 μL of luminol solution containing PMA (final concentration, 1μM) or OZ (final concentration, 1 mg/mL) was added. When fMLF-stimulated LDCL was measured, 40 μL of luminol solution containing fMLF (final concentration, 1μM) was added following incubation with various lactate concentrations and LPS (final concentration, 10 μg/mL) adjusted with equine plasma for 30 minutes at 37°C because equine PMNs require LPS-priming for ROS production with fMLF. Immediately after adding the luminol solution with or without PMA or OZ, the LDCL counts were plotted at 2.5-second intervals for each well and integrated for 30 minutes, except for the 15-minute integration used for stimulation with fMLF. The integrated LDCL was presented as an arbitrary fluorescent unit value and relatively evaluated, compared with LDCL in the absence of lactate as a control.

The potential effect of lactate on ROS scavenging was evaluated with a cell-free assay that used hydrogen peroxide. The assay in the absence of PMNs was performed by the LDCL measurement as well as the unstimulated LDCL condition. The volume of hydrogen peroxide to be added was determined by a preliminary test, in which the LDCL for hydrogen peroxide was equal to the LDCL for unstimulated PMNs incubated in the absence of lactate. The scavenger effect of lactate was presented as the inhibition percentage of the LDCL for hydrogen peroxide.

The priming effect of extracellular lactate on the capacity of PMNs stimulated with PMA, OZ, or fMLF was evaluated as the response ratio, which was partly considered a scavenger effect of lactate on ROS, depending on its dose. To evaluate the response ratio, the LDCL for unstimulated and stimulated PMNs was measured on the same occasion, as described. The response ratio was calculated as follows:

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\text{Response ratio} = \frac{\text{Integrated LDCL in stimulated cells}}{\text{Integrated LDCL in unstimulated cells}}
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The synergic effect of lactate and pH on ROS production by PMNs was evaluated by LDCL with or without PMA (final concentration, 1μM). After incubation of equine PMNs with 0, 30, or 200mM lactate at a pH of 7.4, 7.0, 6.5, or 6.0 for 30 minutes at 37°C, the LDCL was measured for 30 minutes at 37°C.

Statistical analysis—Results are expressed as mean ± SD values derived from samples from 3 to 13 horses. Data were analyzed with the Mann-Whitney U test. Values of P < 0.05 were considered significant as determined by statistical software.

Results

Effect of lactate on equine PMN viability—The viability of equine PMNs was > 99.5% immediately after isolation via the technique described in the present report. When equine PMNs were incubated in the absence or presence of lactate for 30 minutes, their viabilities were 99.0 ± 0.9% in 0mM lactate and 98.5 ± 1.0% in 30, 100, and 300mM lactate, as determined by the trypan blue exclusion test, and 96.7 ± 0.8% in 0mM lactate and 97.7 ± 0.8% in 30, 100, and 300mM lactate, as determined by propidium iodide staining. Incubation with 300mM lactate for 1 hour did not affect the PMN viability (98.6 ± 0.2%) by the trypan blue exclusion test. There was no significant difference between the viabilities of equine PMNs exposed to various lactate concentrations (0, 30, 100, and 300mM), indicating that the results from other experiments were derived from the equal numbers of viable cells.

Lactate-induced shape changes in equine PMNs—Changes in the shape of equine PMNs incubated with various lactate concentrations for 30 minutes were assessed by flow cytometry. When incubated with 0 to 300mM lactate, 87.1 ± 8.7% of PMNs were tightly clustered. These tight clusters of cells were examined to evaluate the potential effect of lactate on shape change. Incubation in the absence of lactate for 30 minutes decreased the size and density of PMNs, compared...
with samples not incubated for 30 minutes (data not shown). Further size reduction and increased density of PMNs occurred with increasing concentrations of lactate (Figure 1). These shape changes were also confirmed by light microscopic analysis. After incubation with various lactate concentrations, the cell diameter of PMNs was reduced, and the chromatin was condensed (Figure 2).

Relative quantification of MCT1, MCT4, LDHA, and LDHB mRNAs—The expression levels of MCT1, MCT4, LDHA, and LDHB mRNAs were quantitatively evaluated via real-time PCR assay. The expression level of MCT1 mRNA did not significantly change and that of MCT4 mRNA was significantly decreased to 0.69-fold and 0.40-fold after incubation with 100 and 200 mM lactate, respectively (Figure 3). The expression level of LDHA mRNA was also significantly decreased to 0.76-fold and 0.53-fold after incubation with 100 and 200 mM lactate, respectively, and the LDHB mRNA level was unaffected.

Effect of lactate on LDCL—Compared with incubation in the absence of lactate, incubation of equine PMNs with 15 to 100 mM lactate significantly decreased the unstimulated LDCL, and incubation with 200 or 300 mM lactate significantly increased the unstimulated LDCL (Figure 4). Thus, the lower concentrations of lactate (up to 30 mM) decreased the unstimulated LDCL, and the higher concentrations of lactate (50 to 300 mM) increased the unstimulated LDCL. The LDCL stimulated with 1 μM PMA, 1 mg of OZ/mL, or 1 μM fMLF was significantly decreased as lactate concentrations increased. In the presence of 100 to 300 mM lactate, fMLF-stimulated LDCL was not substantially detected. Phorbol 12-myristate 13-acetate–stimulated LDCL was decreased by at least 50% with 100 mM lactate, and OZ-stimulated and fMLF-stimulated LDCL was decreased by at least 50% with 30 mM lactate. In the cell-free assay to evaluate the potential effect of extracellular lactate on the scavenging of hydrogen peroxide, the percentage inhibition of LDCL was significantly (P < 0.01) increased in a concentration-dependent manner (Figure 5).

**Figure 1**—Shape changes of equine PMNs incubated with various lactate concentrations. Flow cytometry scatterplots (A to D) indicate the main population clusters in PMN samples (1 X 10^6 cells/mL) that were incubated with 0 (A), 30 (B), 100 (C), and 300 mM (D) of lactate at a pH of 7.4 for 30 minutes at 37°C. The size and density are indicated by forward- and side-angle light scatters, respectively.

**Figure 2**—Photomicrographs of equine PMNs incubated with 0 (left) and 300 mM (right) lactate. Results are representative of the results from 3 horses. May-Grunwald-Giemsa stain; bar = 10 μm.

**Figure 3**—Relative quantification of MCT1, MCT4, LDHA, and LDHB mRNAs. The expression levels of MCT1, MCT4, LDHA, and LDHB mRNAs were quantitatively evaluated via real-time PCR assay. The expression level of MCT1 mRNA did not significantly change and that of MCT4 mRNA was significantly decreased to 0.69-fold and 0.40-fold after incubation with 100 and 200 mM lactate, respectively (Figure 3). The expression level of LDHA mRNA was also significantly decreased to 0.76-fold and 0.53-fold after incubation with 100 and 200 mM lactate, respectively, and the LDHB mRNA level was unaffected.

**Figure 4**—Effect of lactate on LDCL. Compared with incubation in the absence of lactate, incubation of equine PMNs with 15 to 100 mM lactate significantly decreased the unstimulated LDCL, and incubation with 200 or 300 mM lactate significantly increased the unstimulated LDCL (Figure 4). Thus, the lower concentrations of lactate (up to 30 mM) decreased the unstimulated LDCL, and the higher concentrations of lactate (50 to 300 mM) increased the unstimulated LDCL. The LDCL stimulated with 1 μM PMA, 1 mg of OZ/mL, or 1 μM fMLF was significantly decreased as lactate concentrations increased. In the presence of 100 to 300 mM lactate, fMLF-stimulated LDCL was not substantially detected. Phorbol 12-myristate 13-acetate–stimulated LDCL was decreased by at least 50% with 100 mM lactate, and OZ-stimulated and fMLF-stimulated LDCL was decreased by at least 50% with 30 mM lactate. In the cell-free assay to evaluate the potential effect of extracellular lactate on the scavenging of hydrogen peroxide, the percentage inhibition of LDCL was significantly (P < 0.01) increased in a concentration-dependent manner (Figure 5).

**Figure 5**—Priming effect of lactate on the capacity of equine PMNs to produce ROS by stimulants. To evaluate the capacity of equine PMNs to produce ROS, response ratios to PMA, OZ, and fMLF were calculated as the relative values of stimulated LDCL to unstimulated LDCL. Incubation with up to 50 mM lactate did not significantly decrease the response ratio to PMA; the highest response ratio to PMA occurred after incubation with 15 mM lactate (P < 0.05; Figure 6). The response ratio to OZ was not altered by incubation with up to 15 mM lactate. These results for the response ratio to PMA or OZ were different from the results for the LDCL stimulated with PMA or OZ, which had significant decreases after incubation with the same concentrations. Compared with the response ratio in the absence of lactate, a 50% decrease in the response ratio to PMA or OZ occurred in the presence of 100 and 30 mM lactate, respectively. For fMLF stimulus, the pattern of the response ratio was markedly different from the pattern of the stimulated LDCL. The incubation with 15 to 50 mM lactate significantly increased the response ratio to fMLF, compared with the response ratio in the absence of lactate, and the highest response ratio to fMLF occurred with 50 mM lactate. The response ratio to fMLF was significantly decreased by incubation with 200 or 300 mM lactate.
Synergistic effect of lactate and pH on ROS production—The synergistic effect of extracellular lactate and pH on ROS production by equine PMNs was evaluated by unstimulated and stimulated LDCL with 1μM PMA. In the absence of lactate, unstimulated LDCL was significantly increased in a pH-dependent manner (Figure 7). Incubation with 30 and 200mM lactate at a pH of 7.0 to 6.5 and 7.0, respectively, significantly increased unstimulated LDCL, compared with a pH of 7.4. In incubation with 30mM lactate at a pH of 6.0 and with 200mM lactate at a pH of 6.5, unstimulated LDCL significantly decreased, compared with at a pH of 6.5 and 7.0, respectively. In incubation with 200mM lactate at a pH of 6.0, unstimulated LDCL could not be substantially detected. In an assessment of ROS production by PMA-stimulated equine PMNs, stimulated LDCL in the absence of lactate was significantly increased within the pH range from 7.0 to 6.0, compared with at a pH of 7.4. Incubation with 30 and 200mM lactate at a pH of 7.0 significantly increased stimulated LDCL, compared with at a pH of 7.4, and a lower pH of 6.5 or of 6.0 significantly decreased the stimulated LDCL, compared with at a pH of 7.0 and 6.5, respectively.

Discussion

After high-intensity exercise, horses frequently have a blood lactate concentration >30mM (value at rest, 0.6 to 1.7mM) and a muscle lactate concentration of up to 50mM (calculated by converting to mmol/L and assuming that 75% of the muscle composition is water). However, it has remained unknown whether extracellular lactate alters equine PMN functions. Results of the present study indicated that extracellular lactate potentially has a direct effect on the innate immune functions of equine PMNs via the alteration of their morphology, lactate metabolism, and capacity to produce ROS; however, incubation with any lactate concentration for 30 minutes, which is approximately the half-life of lactate in horses,

Figure 3—Relative quantification of gene expression for MCT1 (A), MCT4 (B), LDHA (C), and LDHB (D) mRNAs in equine PMNs incubated with 0, 30, and 200mM lactate at a pH of 7.4 for 30 minutes via real-time PCR assay. Data (mean ± SD) were derived from samples from 3 horses. *Value is significantly (P < 0.05) different, compared with that for cells incubated without lactate.

Figure 4—Effect of lactate on LDCL in equine PMNs. After incubation of PMNs (1 X 10⁶ cells) in the absence (control) or presence of lactate at a pH of 7.4 for 30 minutes at 37°C, unstimulated LDCL (A) was measured for 30 minutes at 37°C. Luminol-dependent chemiluminescence stimulated with 1μM PMA (B) or 1 mg of OZ/mL (C) was measured for 30 minutes after incubation with lactate. Luminol-dependent chemiluminescence stimulated with 1μM FMLF (D) was measured for 15 minutes following incubation with lactate and LPS (10 μg/mL) adjusted with plasma for 30 minutes. Data (mean ± SD) were derived from samples from 11 horses for unstimulated or PMA-stimulated LDCL and 6 horses for OZ- or FMLF-stimulated LDCL. *Value is significantly (P < 0.05) different, compared with the LDCL of PMNs incubated without lactate. AFU = Arbitrary fluorescent unit.
7.4. Differences in experimental conditions or species specificity may explain the differences in results. It is possible that equine PMNs, like human PMNs,\textsuperscript{20} have a higher tolerance to extracellular lactate.

Various factors induce changes in the shape of human PMNs that are a prerequisite for cellular migration and infiltration.\textsuperscript{23} In the present study, lactate incubation uniformly decreased the size and increased the density of equine PMNs (Figure 1 and 2). Lipopolysaccharide incubation of equine PMNs induces increases or decreases in their size for different populations, and both changes in size are accompanied with decreased cell density.\textsuperscript{24} Furthermore, fMLF stimulation of equine and human PMNs primed by LPS induces a shape change toward increased size.\textsuperscript{22} Although the mechanism for the shape change induced by these chemotactic factors remains unclear, the lactate-induced shape change in equine PMNs may be caused by increased osmolality that occurs with increased lactate concentrations. Indeed, increased osmolality decreases the size of human PMNs,\textsuperscript{25} which is consistent with the present study. Further investigation is necessary to determine whether the extracellular lactate–induced shape change of equine PMNs affects the migration and infiltration of these cells. One important finding of the present study was that the extracellular lactate–induced shape change in PMNs occurred under conditions (30-minute incubation with 30mM lactate) that mimic the physiologic state in horses after high-intensity exercise.

In the cell-to-cell lactate shuttle, MCT\textsubscript{1}\textsuperscript{26} and MCT\textsubscript{4}\textsuperscript{27} are correlated to the lactate influx and efflux, respectively, and their presence in human PMNs has been confirmed.\textsuperscript{28} Although MCT1 and MCT4 isotypes are expressed in equine skeletal muscle,\textsuperscript{29} the detailed mechanism of lactate metabolism (including the presence of these isotypes) remains unknown for equine PMNs. In the present study, we found that mRNAs for both MCT isotypes are expressed in equine PMNs as well as human PMNs. Lactate dehydrogenase A and

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Figure 5—Scavenger effect of lactate on hydrogen peroxide in a cell-free assay. After incubation with a solution containing hydrogen peroxide and lactate in 96-well microplates for 30 minutes at 37°C, LDCL was measured for 30 minutes at 37°C in quadruplicate for each concentration. Data (mean ± SD) are presented as percentage inhibition of LDCL for hydrogen peroxide by lactate. *Value is significantly (P < 0.01) different, compared with the value obtained in the absence of lactate.

Figure 6—Effect of lactate on the capacity of equine PMNs to produce ROS in response to stimulants. The capacity of equine PMNs (1 X 10\textsuperscript{6} cells) to produce ROS is presented as the response ratio of LDCL stimulated with 1μM PMA (A), 1 mg of OZ/mL (B), or 1μM fMLF (C) to unstimulated LDCL, following incubation in the absence (control) or the presence of lactate for 30 minutes at 37°C. Data (mean ± SD) were derived from samples from 11 horses for PMA stimulation and 6 horses for OZ or fMLF stimulation. *Value is significantly (P < 0.05) different, compared with the control (0mM lactate).
LDHB isotypes are mainly associated with the reduction (production) and oxidation (degradation) of lactate, respectively. In equine PMNs, MCT1 and LDHB mRNA values were not altered, and MCT4 and LDHA mRNA values decreased significantly after exposure to higher lactate concentrations (100 or 200mM; Figure 3). Although a 30-minute incubation with 30mM lactate could not alter the mRNA values of these isotypes, the present study indicated that these MCT and LDH isotypes play an important role in the metabolization of extracellular lactate in PMNs. Their expression pattern suggests that the incorporation and oxidation of extracellular lactate is greater than the export and reduction of lactate by equine PMNs under this experimental condition.

Oxidation of lactate, which is accompanied by the production of NADH, is associated with increased ROS production via the activation of NAD(P)H oxidase in bovine cardiac myocytes. In equine PMNs, incubation with 100 or 200mM lactate significantly increased the concentration of unstimulated LDCL (Figure 4), suggesting the possibility that the increased LDCL concentration is derived from increased ROS production via lactate oxidation. In contrast, incubation with lower lactate concentrations (15 to 100mM) significantly decreased the concentration of unstimulated LDCL. Extracellular lactate scavenges superoxide, hydroxyl radicals, and hydrogen peroxide of ROS in vitro. In the present study, this effect of extracellular lactate as a potential antioxidant agent was validated by the cell-free assay that used hydrogen peroxide (Figure 5). Furthermore, our finding that the level of LDCL stimulation provided by PMA, OZ, and IMLF decreased according to increased lactate concentrations also supports the scavenger effect of extracellular lactate on ROS production by equine PMNs. Thus, the decrease in unstimulated LDCL at lower lactate concentrations suggests that the ROS scavenging effect is superior to the increased ROS production related to lactate oxidation at physiologic lactate concentrations. In horses recovering from high-intensity exercise, the lactate-dependent scavenging of ROS may contribute to the protection against muscle injury by ROS produced by PMNs or muscle.

In the present study, we found that lactate incubation differentially altered the sensitivity of equine PMNs to PMA, OZ, and IMLF for ROS production. The lactate concentration that reduced the LDCL of equine PMNs by 50% was much higher in receptor-independent PMA stimulation than in receptor-mediated OZ and IMLF stimulation (Figure 4). A similar result was found for the response ratio, which represents the capacity to produce ROS, between PMA and OZ (Figure 6). Reactive oxygen species production induced by receptor-mediated stimuli is more inhibited than receptor-independent PMA in equine and human PMNs incubated with agents that increase the concentrations of intracellular cyclic adenosine monophosphate and hypertonic saline (180 mmol of Na+/L) solution, respectively. Hyperosmolality caused by extracellular lactate might also be a factor responsible for the different sensitivity of equine PMNs to each stimulant, as is the case for human PMNs incubated with hypertonic saline solution.

Results of the present study indicated that lactate incubation had a priming effect on ROS production induced by PMA and IMLF stimuli in equine PMNs (Figure 6). An especially marked priming effect was found in the response ratio of equine PMNs to IMLF; the response ratio was gradually and significantly increased by incubation with up to 50mM lactate, which reflects physiologic concentrations. In human macrophages, lactate transport dependent on MCTs boosts the signaling activation from Toll-like receptor 4, which recognizes LPS, indicating that extracellular lactate promotes the activation of innate immune functions. Furthermore, LPS priming enhances the sensitivity of equine PMNs to IMLF for ROS production in a manner different from human PMNs. On the basis of these 2 findings, we expected that extracellular lactate would prime the capacity to produce ROS in IMLF-stimulated PMNs.

Figure 7—Synergistic effect of lactate and pH on unstimulated and PMA-stimulated LDCL of equine PMNs. Equine PMNs (1 X 10^6 cells) were stimulated with 0 (white bar), 30 (gray bar), or 200mM (black bar) lactate at a pH of 7.4, 7.0, 6.5, or 6.0 for 30 minutes at 37°C, and unstimulated LDCL (A) and stimulated LDCL with 1μM PMA (B) were measured for 30 minutes at 37°C. Data (mean ± SD) were derived from samples from 5 horses. *Value is significantly (% < 0.05) different, compared with the value obtained with a pH of 7.4. †Value is significantly (% < 0.05) different, compared with the value obtained with a pH of 7.0. §Value is significantly (% < 0.05) different, compared with the value obtained with a pH of 6.5. See Figure 4 for remainder of key.
equine PMNs via activation of LPS signaling. This hypothesis was supported by the results, suggesting that extracellular lactate contributes to the regulation of innate immune functions in horses after high-intensity exercise. Poor fMLF-mediated chemotaxis in equine PMNs may also be enhanced by the priming effect of extracellular lactate, as was the case with ROS production in the present study.

Finally, the present study revealed the synergistic effect of extracellular lactate and pH on ROS production by equine PMNs. In human PMNs, incubation with acidic pH medium in the absence of lactate enhances ROS production without a stimulus and with various stimuli (PMA, OZ, and fMLF). In contrast, incubation with acidic pH medium in the presence of lactate reduces PMA, OZ, and fMLF-induced ROS production by human PMNs because their intracellular pH is lowered following the abundant accumulation of lactic acid, which is not caused in the presence of lactate or acidic pH only. An acidic environment induces the activation of human PMNs by a mechanism dependent on the activation of PI3K or Akt and extracellular signal-regulated kinase pathways, and extracellular lactate decreases the PI3K and Akt activity in rat skeletal muscle. The unstimulated and stimulated LDCL in equine PMNs incubated with or without lactate at acidic pH appeared to be altered, as observed in previous reports, suggesting the possibility that their synergistic effect on equine PMN function is regulated by a complex mechanism including the signaling pathways and lactate metabolism. This finding that LDCL values were synergistically increased by the combination of physiologic lactate concentrations and pH also supports the idea that lactate acts as an activator of innate immune functions in horses as well as humans.

Results of the present study suggested that the substantial and prolonged increase of lactate concentrations in horses after high-intensity exercise is associated with short-term alteration of innate immune functions. Our finding that the extracellular lactate affected the scavenging of ROS and altered the different sensitivity to stimulants of ROS production may, at least in part, explain the impairment of ROS production by equine PMNs observed after high-intensity exercise. In contrast, physiologic lactate concentrations increase ROS production via a priming effect, and the synergistic effect with acidic pH on equine PMNs may contribute to the inflammatory responses to bacterial infection and muscle injury after high-intensity exercise. Although further study is needed to determine whether extracellular lactate affects the innate immune functions of horses in vivo, results of the present study will facilitate an understanding of lactate concentrations as a useful indicator for health and fitness in horses after high-intensity exercise.

References

18. Dobos GJ, Burger M, Kuhlmann J, et al. Improved cytosolic free calcium mobilization and superoxide production in bicarbon-

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### Appendix

Primer sequences for relative quantification of MCT1 and MCT4 mRNAs in horses via real-time PCR assay and the 2−∆∆CT method.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′ to 3′)</th>
<th>Position (bp)</th>
<th>Amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCT1_F</td>
<td>CAGCCCTGTGTTCCTGTCTAC</td>
<td>459–479</td>
<td>51</td>
</tr>
<tr>
<td>MCT1_R</td>
<td>CCGAAGAAAGCCTGATTGAG</td>
<td>490–509</td>
<td>51</td>
</tr>
<tr>
<td>MCT4_F</td>
<td>GACACAGCCTGGATCTCCTC</td>
<td>175–194</td>
<td>83</td>
</tr>
<tr>
<td>MCT4_R</td>
<td>CCAAAGCGATTCACACACAC</td>
<td>238–257</td>
<td>83</td>
</tr>
</tbody>
</table>

F and R indicate forward and reverse primers, respectively. Position +1 indicates the start codon, ATG, on the basis of equine MCT1 or MCT4 cDNA sequences (GenBank accession Nos. NM_001081791 and EF564279, respectively). Primer sequences for relative quantification of LDHA, LDHB, and β2M mRNAs have been described.²¹