Equine Neutrophils Respond to PGE_2 by Activating Expression of Core Circadian Clock Genes

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Abstract: An emerging body of evidence supports a relationship between the circadian and immune systems during an innate immune response. Previously, we have demonstrated synchronized upregulation of core circadian clock genes, *Per2* and *Bmal1,* in equine whole blood following lipopolysaccharide (LPS) administration *in vivo*. Subsequent experiments suggested a role for the febrile mediator, prostaglandin E_2 (PGE_2), in mediating this response. However, PGE_2 failed to directly stimulate clock gene expression in equine PBMCs. This study demonstrates that ex vivo cultured equine neutrophils actively respond to PGE_2 by upregulating *Per2* and *Bmal1* expression. In addition, we show that LPS induces marked neutrophilia and concomitant monocytopenia in equine peripheral blood at the time corresponding to the previously observed clock gene rise. We further report that the peak in the PGE_2 mediated endotoxic fever also occurs simultaneously. Combined, our data suggest that neutrophils are the source of the rise in *Per2* and *Bmal1* expression previously observed in equine peripheral blood following LPS administration, and that this response is likely mediated by PGE_2. These results provide the first evidence for a potential role of core circadian clock genes in neutrophil function following innate immune activation.

Keywords: Bmal1, circadian, inflammation, LPS, neutrophil, Per2, PGE_2.

INTRODUCTION

The mammalian circadian system provides a temporal frame necessary to maintain physiological homeostasis by using the varying environmental light/dark cycles to entrain biological processes to a 24-h period. The central pacemaker located in the suprachiasmatic nucleus (SCN) of the hypothalamus receives light information and transmits the timing signal to peripheral tissues, synchronizing temporal expression of core clock genes in individual cells [1-3]. Peripheral clocks have been identified in almost all tissues examined to date and share a similar sequential pattern of clock gene expression as the SCN, exemplified by the antiphase oscillation of *Per2* and *Bmal1* mRNAs in heart, lung, liver, eye, kidney and pancreas [4-6]. In this way, peripheral tissues anticipate environmental changes and can adapt their specific function to the correct time of day by means of tissue-specific circadian regulation of transcription [7-11].

While the circadian system provides a form of predictive homeostasis by activating physiological mechanisms at the time associated with an expected environmental challenge [12], the innate immune system plays an important role in reactive homeostasis by identifying and eliminating invading pathogens that pose a threat to the internal milieu at any time in the 24-h cycle. Inflammation is one of the first responses of the immune system to infection or irritation, and acute systemic inflammation, as occurs in response to the bacterial endotoxin, lipopolysaccharide (LPS), induces profound autonomic, endocrine and behavioral responses that are primarily controlled by the brain [13]. Efficient circadian-immune crosstalk would be of benefit to an organism such that under normal non-inflammatory conditions, the circadian system regulates fluctuations in immune parameters and under acute inflammatory conditions, the reactive immune response stimulates resynchronization of core circadian components, thus in each case maintaining or restoring homeostasis. Recent studies demonstrating circadian regulation of pro-inflammatory cytokines [14, 15], circulating levels of different leukocyte populations [16, 17] and natural killer cell function [18], as well as circadian responses to endotoxin treatment in mice [19], support the existence of such a relationship. In addition, we have demonstrated that LPS synchronizes expression of the core clock genes *Bmal1* and *Per2* in equine whole blood *in vivo* [20].

Based on previous results in mice that demonstrate a clock-resetting property of the febrile mediator prostaglandin E_2 (PGE_2) [21], we previously hypothesized that this pro-inflammatory compound might be the immune modulator responsible for clock gene synchronization in equine peripheral blood during acute systemic inflammation [20]. This idea was further strengthened by the finding that co-administration of LPS and a non-steroidal anti-inflammatory drug (NSAID), which blocked the endotoxin-induced febrile response and

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indicated simultaneous inhibition of PGE_2 production [22], also inhibited the rise in clock gene expression. Subsequent ex vivo studies of clock gene expression in cultured equine PBMCs revealed that neither LPS nor PGE_2 directly induced clock gene upregulation in this leukocyte population [20]. These results suggested that the immune-feedback signal to the circadian machinery in blood cells during an innate immune challenge may be mediated by an indirect pathway, possibly *via* PGE_2 signaling within the central pacemaker in the brain.

However, an alternative interpretation is that a blood cell population other than PBMCs, such as the polymorphonuclear component of blood, is responsible for the synchronized rise in clock gene expression observed during an inflammatory event. Of these, neutrophils are the predominant cell type accounting for 50-60% of all leukocytes in circulation [23, 24]. While it is well known that neutrophils provide the first line of defense against bacterial infections by means of phagocytosis, release of cytoplasmic granule contents and production of reactive oxygen intermediates (ROIs) [25], their genomic response upon activation was traditionally considered to be somewhat static. There is now increasing evidence that neutrophils have a robust transcriptional response to LPS [26, 27], most notably of cytokines, and microarray data has revealed activation of multiple signal transduction systems not previously associated with LPS exposure [28].

Furthermore, as well as inducing profound transcriptional changes in whole blood, LPS also causes dramatic changes in the relative proportions of blood cell populations following endotoxin challenge in rats [29]. Significantly, the authors reported marked neutrophilia and lymphopenia in the hours following LPS administration and suggest that changes in levels of specific transcripts are directly related to leukocyte subset changes.

The ability of LPS to activate equine neutrophils *in vitro* has been well-documented [30, 31]. However, while the *in vivo* response of LPS on circulating equine peripheral blood mononuclear cells has been previously investigated [32], no studies to date have examined the kinetics of circulating polymorphonuclear neutrophils during acute inflammation in the horse. A study of this kind in humans revealed that LPS induces a significant expansion of the neutrophil population in circulation [33]. Therefore, we hypothesize that a similar expansion of the neutrophil population may occur in equine blood in response to LPS.

To determine whether polymorphonuclear cells are responsible for the previously observed clock gene synchronization in equine whole blood following endotoxin challenge, we investigate whether ex vivo stimulation of equine neutrophils with PGE_2 or LPS results in changes in expression levels of the core circadian clock genes, *Per2* and *Bmal1*. In addition, to determine whether the rise in clock gene expression coincides with a rise in neutrophil levels in circulation, we examine the *in vivo* changes in leukocyte populations in equine blood during an acute systemic inflammation.

METHODS

Neutrophil Isolation

Neutrophils were isolated by a previously described method for rapid isolation of polymorphonuclear leukocytes [34] with slight modifications. Peripheral blood from a healthy equine donor was directly drawn into 8 ml Vacutainer cell preparation tubes (CPTs) containing sodium citrate (BD Biosciences, San Jose, CA). The tubes were centrifuged for 25 min at 1700 g at room temperature (RT). The plasma and the PBMCs that were separated above the gel lock were discarded. The upper portion of the gel was washed twice with cold phosphate buffered saline (PBS: 5 ml for each wash). A 3 mL syringe was attached to an 18 gauge 1.5 in. needle and then pierced through the gel to collect the erythrocyte/neutrophil mixture into the syringe. The needle was removed and the cells were collected into 50 ml centrifuge tubes. The cells were washed with cold Ca^{+2} and Mg^{+2} - free PBS (Sigma, Saint Louis, Missouri) containing 2.5 % autologous serum by centrifugation for 10 min at 400 g at 4 °C. The erythrocytes were lysed with EL buffer (Qiagen, Valencia, CA) for 10 min at RT with frequent vortexing of the tubes. The cells were washed twice with cold Ca^{+2} and Mg^{+2} - free PBS by centrifugation for 10 min at 400 g at 4 °C. Cells were then suspended in RPMI 1640 medium (Gibco, Invitrogen Corporation, Grand Island, NY) supplemented with 2.5 % autologous serum, 100 U/mL streptomycin/penicillin and 2 mM L-glutamine (Sigma). Cells were counted and checked for viability by trypan blue exclusion on a Vicell^{TM}XR Cell Viability Analyser (Beckman Coulter, Fullerton, CA). Neutrophil purity was determined by flow cytometry. For this, cells were indirectly stained with monoclonal antibodies (mAbs) directed against equine CD172 (clone DH59B, VMRD, Pullman, USA), CD14 (clone big 10, Biometec, Grefswald, Germany) or CD5 (courtesy of Dr. D.P. Lunn, University of Colorado) or with a control mAb of the same isotype (MOPC-21, mouse IgG1, SIGMA). The specificity of the mAbs has been previously demonstrated (Steinbach *et al*., 2005; Tumas *et al*., 1994; Lunn *et al*., 1991). Cells (500,000) were incubated with the mAbs (500 ng, unless specified by the manufacturer) for 30 min at 4˚C and washed twice in cold Ca^{+2} and Mg^{+2} - free PBS at 300 g for 5 min. After 15 min of incubation with 500 ng of FITC-labeled $F(ab')_2$ fragments of goat anti-mouse Ig (H+L) (Caltag), the cells were washed twice, fixed in 1% paraformaldehyde and analyzed by flow cytometry as described below for whole blood.

Neutrophil Culture and RNA Isolation

Neutrophils (4 X 10^6 cells/mL) were left untreated or treated with LPS (1 μg/mL) or PGE_2 (100 nM, 1 and 10 μM) and incubated in suspension for 4 h at 37 °C in a 5 % CO_2 /air mixture. Following incubation, cells were washed with Ca^{+2} and Mg^{+2} - free PBS and total RNA was isolated using the RNeasy® mini kit (Qiagen) with an additional on-column DNA digestion using the RNase-Free DNase set (Qiagen). Total RNA was quantitatively assessed using the NanoDrop® ND-1000 Spectrophotometer (Agilent Technologies, Palo Alto, CA).

Real Time RT-PCR

Equine *Per2, Bmal1, TNFα* and *β-Actin* were identified, isolated and Taqman primers and probes designed as previously described [35] with the exception of *β-Actin*. The relevant *β-Actin* sequences are: forward primer 5 ‘ -gccgtcttcccc tccat- 3’; reverse primer 5 –gcccacgtatgagtccttctg- 3’: probe 5’ –ggcaccagggcgtgatggtgggc- 3’. Taqman quantitative Real-

Time RT-PCR was performed using a Smart Cycler Real-Time thermal cycler (Cepheid, Sunnyvale, CA) to detect transcript levels of these equine genes in neutrophils. The Taqman system allows the detection of increasing amounts of amplicons at every PCR cycle. The efficiency of primer/probe combinations was tested by running serial 10-fold cDNA dilutions. The correlation between the Ct value (the number of PCR cycles required for the fluorescent signal to reach a threshold level) and the amount of cDNA standard was linear over a 5-log range. Each 25 µl reaction contained 1 x EZ buffer (Applied Biosystems, Foster City, CA), 300 µM of each dNTP, 2.5 mM manganese acetate, 200 nM forward and reverse primer, 125 nM fluorogenic probe, 40 U RNasin (Roche, Indianapolis, IN) and 2.5 U rTth (Applied Biosystems). Cepheid also recommends the addition of an 'Additive Reagent' to prevent binding of polymerases and nucleic acids to the reaction tubes. This reagent was added to give a final concentration of 0.2 mg/ml bovine serum albumin (non-acetylated), 0.15 M trehalose and 0.2 % Tween 20. Thermocycler parameters consisted of a 30-min reverse transcription (RT) step at 60°C, 3 min at 94°C and 40 cycles of: 94°C for 15 s (denaturation) and 60°C for 30 s (annealing and extension). In the case of each sample, quantitative measurement of the level of transcripts of the housekeeping gene product *β-Actin* was used as an internal control of sample-to-sample differences in RNA concentration. *β-Actin* was first tested for its suitability as an endogenous control in equine neutrophils by confirming that its expression did not vary significantly across treatments. Gene expression levels are reported as the number of transcripts per number of *β-Actin* molecules and are expressed as a percentage of the peak value.

Animals

Four healthy mares (*Equus caballus*) of mixed light horse breed ranging in age from 3 to 4 years were used in this study. These were the same 4 mares that were used in a previous study demonstrating clock gene upregulation in peripheral blood in response to endotoxin induced acute systemic inflammation [20]. Two days prior to the current experiment mares were housed indoors in individual stalls and allowed ad libitum access to hay and water. Beginning at 7am on the control day, blood samples were collected by jugular venipuncture and core body temperature (CBT) readings were determined per rectum at 4-h intervals for 12 h. 48 h later, 0.045 µg/kg LPS was administered i.v. following baseline samples at 7am. This dose was chosen based on previous experiments in our laboratory and previously published equine studies demonstrating the effectiveness of this dose at approximating naturally occurring endotoxemia [36-38]. Blood samples were collected and CBT readings recorded 1, 4, 8 and 12 h post LPS treatment. An additional CBT reading was taken at 2 h post LPS in order to more closely monitor the condition of the animals. All procedures involving animals were approved by the University of Kentucky's Institutional Animal Care and Use Committee (IACUC).

Hematology

Blood samples were collected into EDTA Vacutainer tubes (BD Biosciences) and measured for total and differential leukocyte counts using the Idexx QBC VetAutoread hematology system (Idexx Laboratories, INC., Westbook, ME) within 30 min of collection. This machine is a quantitative buffy coat analyzer and separates cells using centrifugation followed by measurement of the different heights of each cell layer within a microhaematocrit tube. The buffy coat layer is expanded using a small float, and a fluorescent dye stains the cells to aid differentiation [39]. Changes in neutrophil and lymphocyte/monocyte cell populations as well as total WBC (white blood cell) counts are reported as millions of cells/mL.

Whole Blood Immunostaining and Flow Cytometry Analysis

The changes in leukocyte populations were assessed by flow cytometry following whole blood staining. 50 µl of blood were incubated for 15 min at room temperature with the same mAbs as for neutrophils. The red blood cells were then lysed with EL buffer (Qiagen, Valencia, CA) at RT for 10 min. The blood cells were centrifuged for 5 min at 540 g and washed in PBS in the same conditions. Following 15 min of incubation with the FITC-anti-mouse antibody, they were washed twice in PBS at 300 g and fixed in 1 % paraformaldehyde. The cells were kept at 4°C before flow cytometry data acquisition within 24 h. Prior to each acquisition process, a FacsCalibur flow cytometer (BD Biosciences) was calibrated with standard beads (Calibrite 3, BD) using the Facs Comp Software. Data from 50,000 events were recorded for each staining, using the Cell Quest Pro Software (BD Biosciences), with the same settings for every sample. The percentage of leukocyte populations was determined by their surface expression of CD172 (neutrophils and monocytes), CD14 (monocytes) or CD5 (lymphocytes).

Statistical Analysis

One-way analysis of variance (ANOVA) followed by Dunnett's post hoc test for comparisons to a control were used to assess variation in gene expression between treatments in cultured neutrophils. Differences in leukocyte population and CBTs were determined by two-way (time x treatment) repeated-measures ANOVA followed by Bonferroni post hoc tests. Data were analyzed using Graph Pad Prism Version 4.0 for Windows (Graph Pad Software, San Diego, CA) and are presented as the means ± SEM. A value of $p < 0.05$ was considered significant.

RESULTS

Per2, Bmal1 and TNFα Expression in Neutrophils Treated with LPS or PGE_2

The cultured neutrophil population was determined as greater than 90 % pure by flow cytometry (Fig. **1**). *Per2* expression in equine neutrophils increased significantly ($F(13) = 4.62$, $p < 0.05$) following a 4 h incubation with 10 µM PGE_2 (Fig. **2**). Expression of this gene was unaffected by treatment of cells with LPS or a lower dose of PGE_2. Similarly, *Bmal1* expression increased significantly ($F(13) = 8.59$, $p < 0.01$) following treatment of neutrophils with 10 µM PGE_2. Again, expression of this clock gene were unaffected by treatment with LPS or a lower dose of PGE_2. However, LPS treatment of neutrophils resulted in significant ($F(13) = 18.34$, $p < 0.001$) upregulation of the pro-inflammatory cytokine *TNFα*. Changes in gene expression of all three genes are shown in Fig. (**2**).

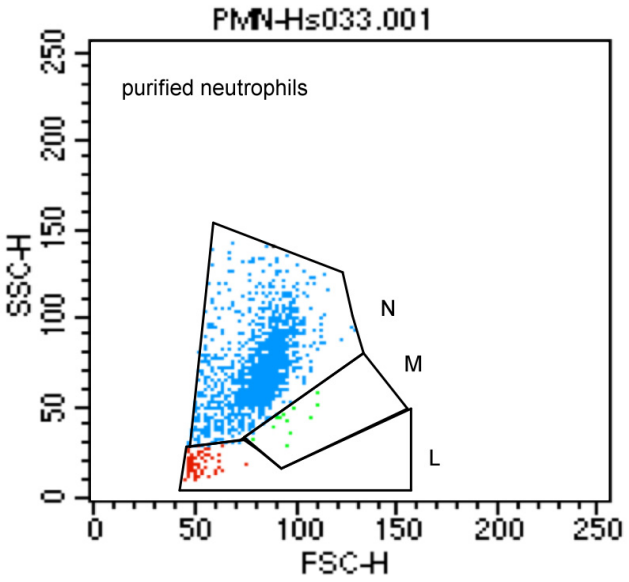

Fig. (1). Assessment of neutrophil purity by fluorescent immunostaining. Cells were stained with anti-equine CD172, CD14 and CD5 mAbs and analyzed by flow cytometry. Back-gating with these stainings indicates that the isolated cells were neutrophils (N), in contrast to whole blood cells (see Fig. **5**). M: monocytes, L: lymphocytes.

Leukocyte Population Changes During Endotoxin Induced Acute Systemic Inflammation in Equine Blood

Acute systemic inflammation resulted in marked neutrophilia and lymphocytopenia in equine blood (Fig. **3**; Table **1**). Absolute neutrophil counts, as determined by hematology analysis, initially decreased by 23 % from control levels at 1 h post LPS treatment and then rapidly increased to 35 %, 108% and 69 % greater than control levels at 4 h, 8 h and 12 h, respectively. Combined lymphocyte/monocyte (L/M) levels decreased by 70 % at 1 h post LPS treatment and remained at 47 %, 45 % and 17 % below control levels at 4 h, 8 h and 12 h, respectively. Overall, total WBC counts dropped by 50% at 1 h post treatment, returned to control levels at 4 h and increased to 38 % and 29 % greater than controls at 8 h and 12 h, respectively (Fig. **4**). There were no significant differences between neutrophil and L/M counts at any time point on the control day. In contrast, two-way repeated measures ANOVA revealed a significant time x treatment interaction ($F(12,4) = 20.11$, $p < 0.0001$) and significant effect of time ($F(4) = 22.33$, $p < 0.0001$) and treatment ($F(3) = 25.85$, $p < 0.0001$) on neutrophil and L/M levels following LPS. Post hoc tests revealed significant ($p < 0.001$) differences between groups at each time point following the baseline values at 0 h. These results were supported by almost identical results using the flow cytometry method of cell count determination. Slight variations in absolute cell counts between methods were not reflected in any differences in statistical significance. As shown in Fig. (**5**, whole blood dot plots), the neutrophil population was reduced 1 h after LPS injection, but expanded 4 h and 8 h post LPS. In contrast, the mononuclear cell pool (lymphocytes and monocytes) was still reduced at 4 h, 8 h and 12 h. Absolute neutrophil, lymphocyte and monocyte counts calculated from flow cytometry percentages confirmed that LPS induced significant neutrophilia (data not shown).

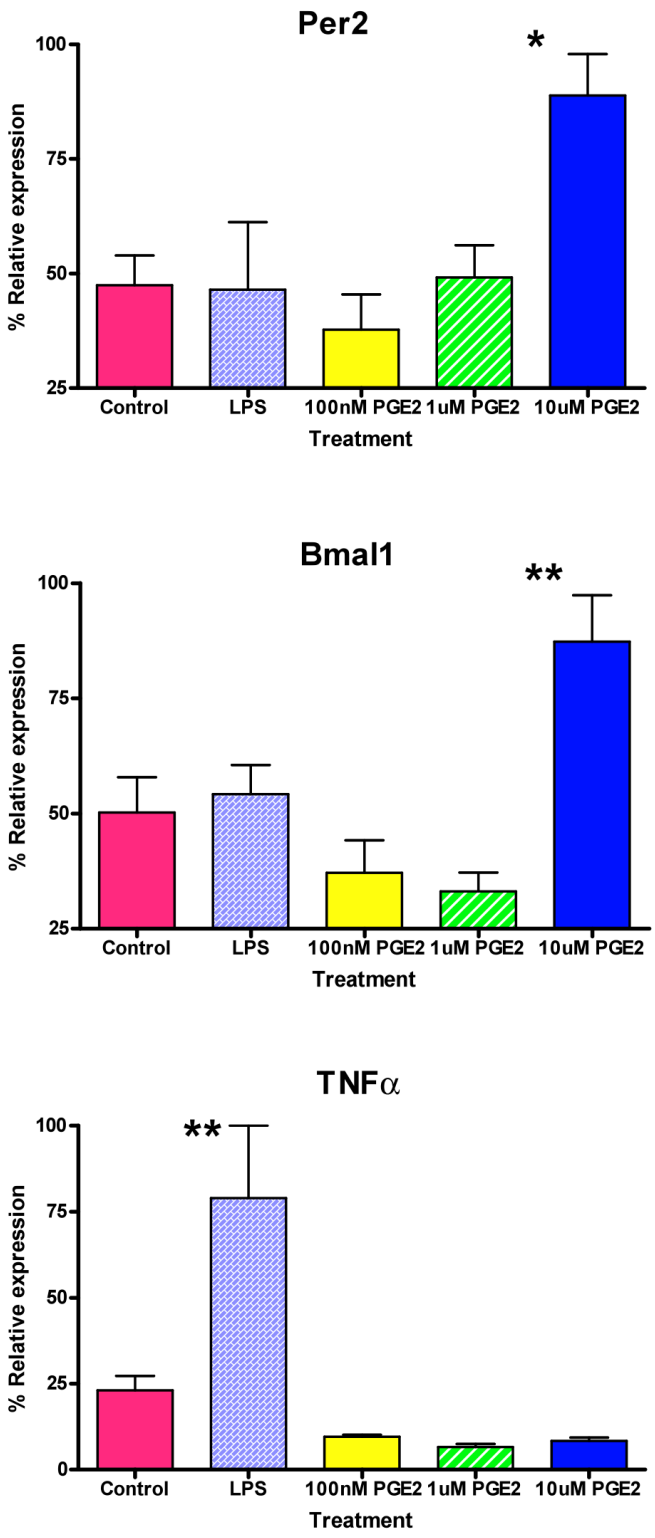

Fig. (2). mRNA expression levels of *Per2*, *Bmal1* and *TNFα* in equine neutrophils cultured for 4 h in media alone, or supplemented with 1 μg/mL LPS or 100 nM, 1 μM and 10 μM PGE$_2$. Gene expression levels are expressed as the number of transcripts per number of molecules of the internal control gene *β-actin* and are reported as a percentage of the peak value. The data are represented as means ± SEM of three independent experiments. *, ** denote significant difference ($p < 0.05$, $p < 0.01$, respectively) from control values.

Table 1. Mean Absolute Values of Leukocyte Differentials

	Control[1] 0 h	LPS[1] 0 h	Control[1] 1 h	LPS[1] 1 h	Control[1] 4 h	LPS[1] 4 h	Control[1] 8 h	LPS[1] 8 h	Control[1] 12 h	LPS[1] 12 h
Total WBC[2]	10±.4	10.3±.3	9.9±.4	4.9±1.3	8.8±.6	8.5±.9	9.2±.6	12.7±.4	9.8±.7	12.7±.8
Neutrophils	5.1±.4 (51)	5.2±.4 (50.4)	5.1±.4 (55.5)	3.9±.7 (79.6)	4.8±.4 (54.5)	6.4±.8 (75.3)	5±.5 (54.4)	10.4±.4 (81.9)	5.2±.5 (53)	8.8±.7 (69.3)
L/M[3]	4.6±.1 (46)	5.1±.3 (49.5)	4.5±.1 (45.5)	1.4±.5 (28)	4±.3 (45.5)	2.1±.1 (24.7)	4±.1 (43.5)	2.3±.2 (18)	4.6±.2 (46.9)	3.8±.1 (29.9)
Number	4	4	4	4	4	4	4	4	4	4

[1]All values, except number, are means ± SEM and represent 10^6 cells/ml. Numbers in parentheses are percentages of total white blood cell count.
[2]White blood cells.
[3]Lymphocytes and monocytes.

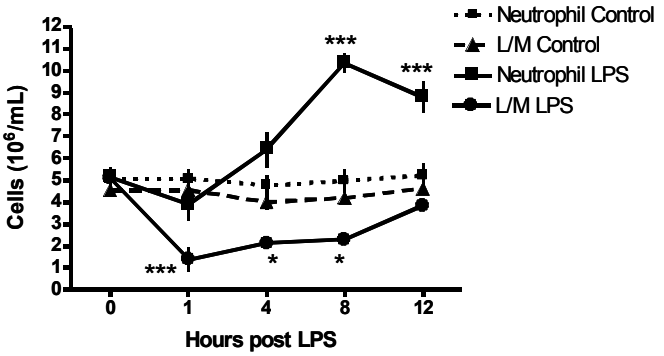

Fig. (3). Peripheral blood leukocyte changes over time in control and LPS treated horses as determined by hematology analysis. The data are expressed as millions of cells per mL of blood and are represented as means ± SEM (n= 4) per time point. L/M: Combined lymphocyte and monocyte counts. *, *** denote significant difference (p <0 .05, p <0.001, respectively) from control values at corresponding time points.

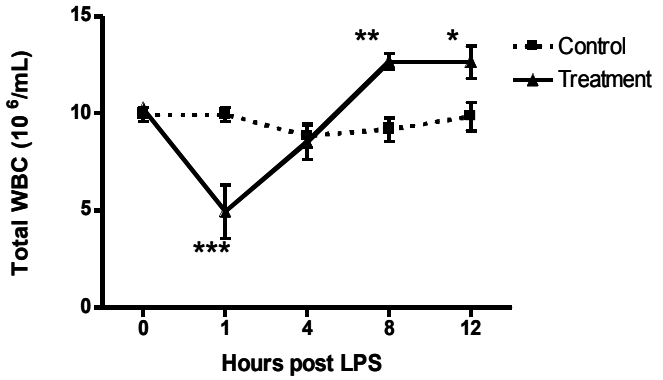

Fig. (4). Peripheral blood total white blood cell (WBC) count changes in control and LPS treated horses as determined by hematology analysis. The data are expressed as millions of cells per mL of blood and are represented as means ± SEM (n= 4) per time point. *, **, *** denote significant difference (p < 0.05, p < 0.01, p < 0.001, respectively) from control values.

Core Body Temperature (CBT) Changes During Endotoxin Induced Acute Systemic Inflammation

Changes in CBT over a 12-h period on the control and LPS treatment days are shown in Fig. (**6**). There was a significant time x treatment interaction ($F(5,1) = 10.42$, p <0.0001) and a significant effect of time ($F(5) = 3.65$, p < 0.05) and treatment ($F(1) = 19.6$, $p < 0.001$). LPS treatment resulted in the expected febrile episode beginning 1 h post treatment and peaking at 4 h. Post hoc tests revealed significant differences between groups at 2h and 4 h ($p < 0.01$ and $p < 0.001$, respectively).

DISCUSSION

PGE_2 Stimulates Upregulation of Per2 and Bmal1 in Equine Neutrophils

In contrast to other species, there is no evidence of synchronized clock gene expression in equine peripheral blood from normal, healthy animals [35]. However, induction of an acute inflammatory response by administration of LPS stimulates synchronous elevated transcription of *Per2* and *Bmal1* that reaches maximal levels 4 h post-treatment [20]. Inhibition of the rise in *Per2* and *Bmal1* expression by co-administration of the NSAID, phenylbutazone, a known suppressor of cyclooxygenase-2 synthesis in equine tissues [40], suggested that the effects of LPS on clock gene transcription in equine blood were mediated through PGE_2, rather than through Toll-like receptor-4 complexes. This conclusion is not without precedent since PGE_2 has been demonstrated to induce or reset circadian rhythms in clock gene expression in mouse fibroblast (NIH3T3) cells and mouse peripheral tissues [21]. The results presented here support the hypothesis that the rise in *Per2* and *Bmal1* expression observed in equine peripheral blood following LPS administration is mediated by PGE_2. Addition of this pro-inflammatory compound to enriched populations of equine neutrophils induced transcription of *Per2* and *Bmal1,* whereas LPS did not. While this effect was only observed for the higher PGE_2 concentration, this likely reflects *in vitro* conditions that do not adequately mimic an *in vivo* inflammatory response. The accelerated rate of neutrophil apoptosis associated with inflammatory conditions *in vivo*, facilitated by direct interaction with pathogen and other cells involved in the inflammatory response [41, 42], may have a synergistic effect on clock gene upregulation at lower physiological PGE_2 concentrations. This would explain the robust rise in clock gene expression previously observed in response to LPS *in vivo* [20]. In fact, results from preliminary *in vitro* studies demonstrated significantly greater fold increases in clock gene expression in response to PGE_2 when associated with reduced percent neutrophil viability prior to treatment (unpublished results).

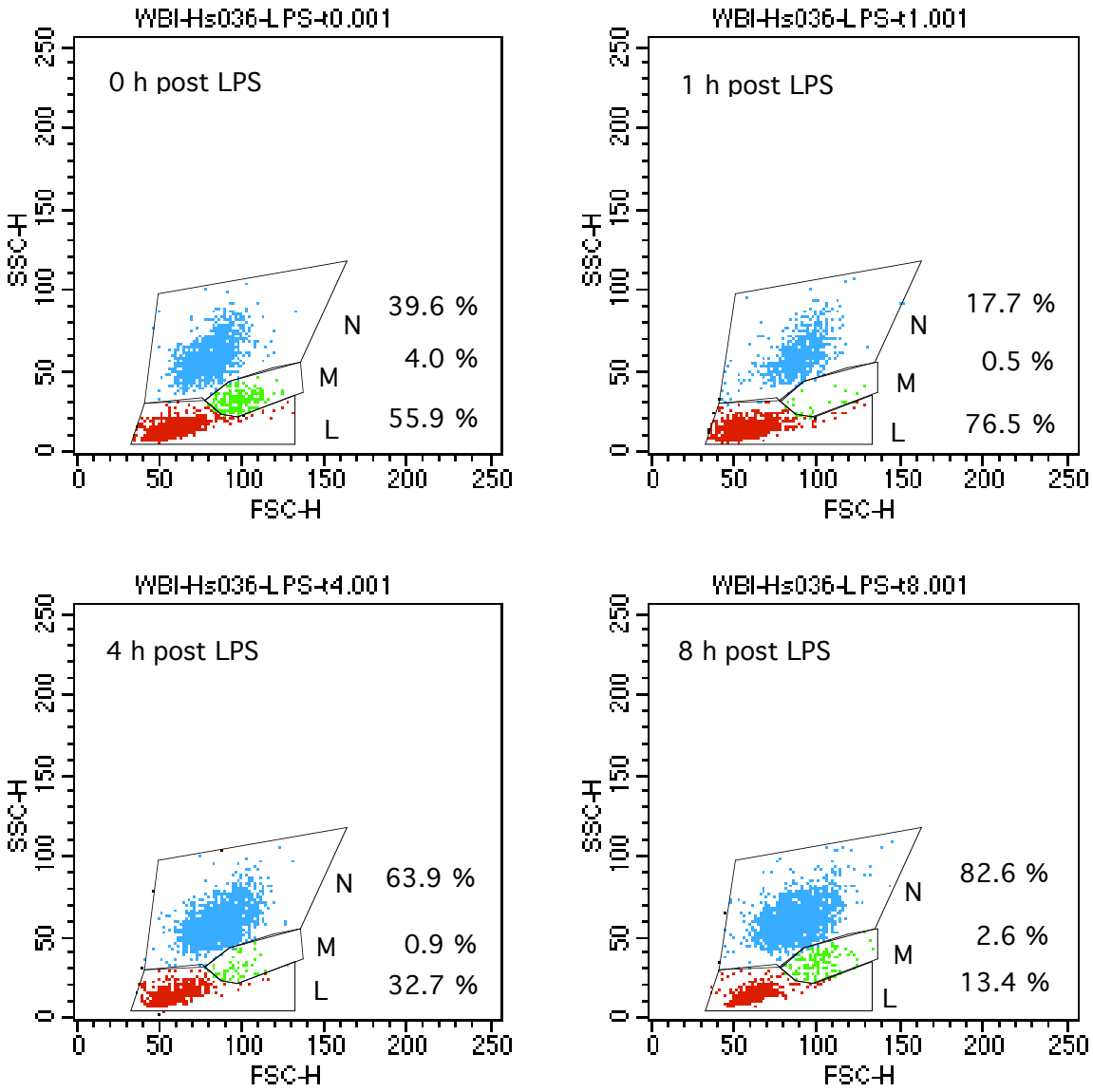

Fig. (5). Flow cytometry analysis of the changes in the leukocyte composition of equine whole blood after administration of LPS. Whole blood was stained with anti-horse CD172, CD14 and CD5 mAbs. Cells were gated according to their expression of CD172 (neutrophils, monocytes), CD14 (monocytes) or CD5 (lymphocytes). The positivity of the labeling was determined by comparison with the staining given by an irrelevant mouse IgG1. N: neutrophils, M: monocytes, L: lymphocytes. Percentages of total population per gate are provided.

Furthermore, upregulation in *Per2* and *Bmal1* mRNA synthesis in PGE_2 treated neutrophils occurred simultaneously, similar to our previous observations in whole blood [20]. This is in contrast to the normal temporal profiles observed in the SCN and most peripheral tissues (including equine adipose tissue), where an antiphase expression of these genes occurs [4-6]. However, in-phase oscillation of *Per2* and *Bmal1* was also recently revealed in human blood [43] and it was suggested that the absence of a neural communication pathway between the SCN and peripheral blood may explain the altered circadian regulation of clock gene expression in this tissue. Although LPS had no measurable effect on clock gene expression in enriched equine neutrophil cultures, it did cause a significant increase in *TNFα* mRNA levels. This supports recent findings that these short-lived cells are transcriptionally active [28] and contribute more to innate immune responses than just the release of pre-made inflammatory mediators [26, 27].

Core Body Temperature And Leukocyte Population Changes in Response to LPS *In Vivo*

The LPS induced rise in core body temperature reached its peak at 4 h post-treatment and therefore corresponded with maximal *Per2* and *Bmal1* expression levels observed in equine blood in our previous experiments using the same animals [20]. PGE_2 is required for the induction and maintenance of febrile responses [44-47]. As circulating concentrations of this molecule are closely correlated with increases and decreases in core body temperature [45, 48], it is expected that concentrations of PGE_2 in blood would be at their highest 4 h post-LPS treatment.

In addition to inducing fever, LPS treatment had profound effects on the numbers and relative proportions of different equine blood cell populations. These changes in leukocyte population kinetics were found to be almost identical to those observed following LPS administration in hu-

mans [33]. In both species, LPS caused a pronounced neutrophilia with concomitant decreases in PBMCs. Although the neutrophilia did not peak until 8 h, at 4 h post-LPS treatment, when circulating levels of PGE_2 are likely to be highest, neutrophils were 35 % higher and PBMCs 53 % lower compared to control levels. Therefore, observations from this and our previous study demonstrating the effects of phenylbutazone [20], provide convincing evidence that following an acute inflammatory stimulus, PGE_2 acts directly on neutrophils to trigger the synchronous, enhanced expression of *Per2* and *Bmal1*. Furthermore, detection of this effect is likely amplified by LPS induced expansion of the circulating neutrophil population.

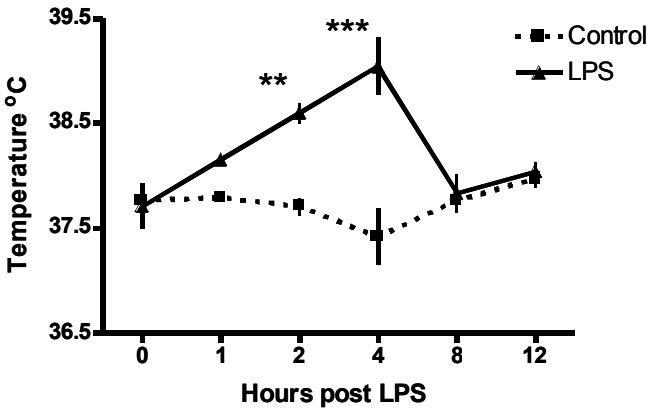

Fig. (6). Changes in core body temperatures (CBT) over a 12-h period in control and LPS treated horses. The data are represented as means ± SEM (n= 4) per time point. **, *** denote significant difference ($p < 0.01$, $p < 0.001$, respectively) from control values.

These results provide the first evidence for a potential role of core circadian clock genes in neutrophil function following activation of innate immune responses. This is not the first study to identify a role for clock genes in immune cell function. Recent evidence also highlighted an essential role for *Per2* in gamma interferon release from natural killer (NK) cells during LPS induced endotoxic shock in mice [49]. While the function of clock gene upregulation in neutrophils remains to be further characterized, PGE_2 is currently known to regulate a range of functions in human neutrophils including chemotaxis [50], superoxide anion generation [51, 52], apoptosis [53, 54] and differential cytokine production [55]. It is now conceivable that clock gene expression may be involved in some or all of these processes. In this regard it is important to note that the CLOCK protein, which is the heterodimerization partner of BMAL1, has been shown to function as a histone acetyltransferase with chromatin remodeling activity that is significantly enhanced when bound to BMAL1 [56]. Therefore, PGE_2 mediated upregulation of *Bmal1* expression likely induces epigenetic changes in neutrophil chromosomal DNA resulting in altered patterns of gene transcription.

The results of these experiments bring to light a new relationship between the circadian and immune systems in the horse in response to a homeostatic challenge and highlight the importance of further investigation of neutrophil function during an innate immune response.

ABBREVIATIONS

Bmal1	=	Brain-muscle-ARNT-like protein 1
CBT	=	Core body temperature
LPS	=	Lipopolysaccharide
mAb	=	Monoclonal antibody
PBS	=	Phosphate buffered saline
PBMC	=	Peripheral blood mono-nuclear cell
Per2	=	Period 2
PGE_2	=	Prostaglandin $(E)_2$
TNFα	=	Tumor necrosis factor alpha
RT-PCR	=	Reverse transcription polymerase chain reaction

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