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In vivo endotoxin synchronizes and suppresses clock gene expression in human peripheral blood leukocytes

Beatrice Haimovich, PhD, **Jacqueline Calvano, MS**, **Adrian D. Haimovich**, **Steve E. Calvano, PhD**, **Susette M. Coyle, MS, RN**, and **Stephen F. Lowry, MD**
Department of Surgery, Division of Surgical Sciences, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, New Brunswick, NJ 08903. USA

Abstract

Objectives—The intravenous administration of a bolus dose of endotoxin to healthy human subjects triggers acute systemic inflammatory responses that include cytokine production and dynamic changes in gene expression in peripheral blood leukocytes (PBL). This study sought to determine the state of clock gene expression in human PBL, and leukocytes subpopulations, challenged with *in vivo* endotoxin at two circadian/diurnal phases of the clock.

Design—Clinical and laboratory investigation.

Setting—University-based research laboratory and clinical research center

Subjects—Human volunteers.

Interventions—Human subjects were administered a standard dose of endotoxin (2ng/kg) or saline at either 09:00 or 21:00 h. Blood samples were collected at selected time points pre- and post-infusion.

Measurements and Mains results—Clock gene expression was determined in human PBL, neutrophils, and monocytes, by quantitative real-time polymerase chain reaction. The fold change for each gene was determined using the $2(^{-\Delta\Delta Ct})$ method. We show that endotoxin causes profound suppression of circadian clock gene expression, clearly manifested in human PBL, neutrophils, and monocytes. *Clock, Cry1-2, Per3, CSNK1ε, Rora* and *Rev-erb* gene expression were all reduced by 80-90% with the nadir between 3 to 6 hours post-infusion. *Per1* and *Per2* reached an expression nadir between 13 and 17 hours post-infusion. The levels of plasma interleukin-6 and tumor necrosis factor peaked and then returned to baseline within 6 hours. In contrast, clock gene expression remained suppressed for up to 17 hours, irrespective of the phase of the clock at the time of the endotoxin challenge. Endotoxin did not perturb the melatonin secretory rhythm.

Conclusions—Circadian clock gene expression in PBL is dramatically altered, and possibly uncoupled from the activity of the central clock, during periods of acute systemic inflammation. The realignment of the central and peripheral clocks may constitute a previously unappreciated key factor affecting recovery from disease in humans.

Keywords

endotoxin; circadian clock; peripheral blood leukocytes; melatonin

Corresponding authors: Beatrice Haimovich, haimovic@umdnj.edu, Telephone: 732-235-8049, Fax: 732-235-6003; Stephen F. Lowry, lowrysf@umdnj.edu, Address: Department of Surgery, Clinical Academic Building, 125 Paterson Street, Suite 7300, New Brunswick, NJ 08903, Telephone: 732-235-6096, Fax: 732-235-6003.

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Introduction

Numerous physiologic activities in higher organisms are synchronized with the 24 h rotation period of the Earth. The central "master" clock controlling behavioral circadian rhythms is located in the suprachiasmatic nucleus (SCN) within the brain hypothalamus [1,2]. The molecular components of the circadian clock consist of transcription factors and proteins whose activity and/or availability cycle with a periodicity of approximately 24 h [3-5]. Circadian oscillations of clock-associated genes have been identified in many tissues (e.g., [6-9]), highlighting the probable existence of multiple peripheral clocks. The mechanisms by which the master clock and the peripheral clocks interact are not completely understood.

Circadian rhytmicity and host immunity are closely interrelated. The number of circulating red blood cells, platelets, as well as all human peripheral blood mononucleated cells subsets exhibit significant diurnal variation [10,11]. Lange et al [12] reported that human monocytes obtained during the normal sleep hours produce interleukin-12 (IL-12) in response to ex-vivo endotoxin challenge, whereas monocytes obtaining during the day produce IL-10. There is also evidence that intracellular IL-6 expression exhibits distinct diurnal pattern, with increasing IL-6 levels during the night. [13]. These findings suggest that both immune cell number and function are subject to circadian regulation.

The core of the circadian clock is composed of two transcription factors, *Clock* and *Bmal1* (*Arntl; MOP3*), which form a heterodimeric complex. Analyses of mice with genetic alterations in *Bmal1* and *Clock* provided further insight into the relationship between immunity and the circadian clock. *Bmal1-/-* mice exhibit no locomotor rhytmicity and are significantly less active than normal mice [14]. In addition, *Bmal1-/-* mice exhibit increased segmented neutrophils and platelets numbers, and decreased B lymphocytes numbers [15]. The daily rhythmic variability in white blood cell populations, and the total number of white blood cells, red blood cells, and platelets were similarly reduced in mice that express a transcription-deficient mutant form of *Clock* [16].

Data suggest that the immune system is not only subject to circadian regulation, but may also provide inputs that alter the circadian activity of the clock(s) [17]. It is also known that activities such as sleep, movement, and food intake that are normally regulated by the circadian clock, are modified during periods of systemic inflammation [18,19]. Tumor necrosis factor (TNFα, IL-1β, and IL-6 are among the prominent pro-inflammatory cytokines produced in human during the acute period of systemic inflammation. Cavadini et al [20] reported that infusion of TNFα in mice triggered a significant reduction in locomotor activity, prolonged rest time, and impaired expression of several clock-related genes in hepatic tissue. The authors proposed that TNFα-induced behavioral changes were triggered, at least in part, through alterations in clock gene expression [20]. These observations underscore the possibility that pro-inflammatory cytokines produced by activated immune cells have the capacity to reset the circadian clock in peripheral tissues during inflammation.

The status of circadian clock genes in humans during periods of systemic inflammation is largely undetermined. Okada et al [21] recently reported that the administration of endotoxin to rats impaired the expression of the clock genes *Per1*, *Per2*, and *DBP* in the liver, and *Per2* and *DBP* in the SCN, with the expression nadir between 10-14 hours post-endotoxin challenge. The objective of this study was to determine whether clock gene expression is perturbed in human PBL during the acute period of systemic inflammation induced by an *in vivo* endotoxin (LPS) challenge [22,23]. Our data revealed that *in vivo* endotoxin causes profound and concurrent suppression of many clock genes in PBL with a nadir between 3-6 hours post-infusion. These observations identify endotoxin as a potent and acute entrainer of the circadian clock gene network in PBL in humans.

Materials and Methods

Study population and procedures

Male and female subjects (age 18-29) were recruited by public advertisement to participate in a study approved by the Institutional Review Board of the Robert Wood Johnson Medical School. Subjects were administered a standard dose of endotoxin (2 ng/kg, NIH Clinical Center Reference Endotoxin, CC-RE, Lot 2, Bethesda, Maryland) [24] or saline (0.9% sodium chloride). Additional information is provided in the Supplementary Materials.

Gene expression analyses in PBL

Blood was drawn into Paxgene™ tubes (PreAnalytix). Total RNA was extracted as per PAXgene Blood RNA kit protocol (Qiagen), quantified using Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA), and reversed transcribed to cDNA using High capacity cDNA Archive kit (Applied Biosystems). Gene expression was analyzed in duplicates by quantitative real-time (qRT) PCR using inventoried TaqMan® gene expression assays (Applied Biosystems). A list of the gene expression assays is provided in the Supplementary Materials. The relative gene expression analysis was performed using the $2^{-\Delta\Delta CT}$ method [25]. The level of *beta-2-microglobulin (B2M)* expression was used as an internal reference, since the expression of *B2M* is not affected by endotoxin administration in vivo [24, 26, 27]. Data are expressed as fold change relative to time 0. For the sample at time 0, $\Delta\Delta C_T$ equals zero and 2^0 equals one, so that the fold change in gene expression relative to the control equals one [25].

Gene expression analyses in leukocyte cell subpopulations

Detailed protocols can be found in the Supplement.

Cluster analysis

Details can be found in the Supplementary Materials. In brief, the data were clustered into a tree form using the standard hierarchical Unweighted Pair Group Method with Arithmetic Mean algorithm (UPGMA), with one minus the cross-correlation coefficient as a measure of similarity [28, 29]. All clustering operations were carried out in MatLab 2008a program.

TNFα, IL-6, and cortisol levels were determined as described [30,31].

Melatonin

Plasma melatonin levels were determined using a direct Melatonin radioimmunoassay kit (Rocky Mountains Diagnostics, Colorado Springs, CO 08903). The assay range was 0-1000 pg/ml.

Statistics

Analysis of three groups or more was by one-way ANOVA with Newman-Keuls post-test. Two groups were compared by unpaired Student's *t* test. The operations were carried out using Prism 4 software Version 4.0b (GraphPad Software, Inc., La Jolla, CA). *P* values less than 0.05 were considered to be statistically significant.

Results

Day study

Subjects were administered endotoxin (n=4) or saline (control; n=2) at 09:00 h (time 0). The relative gene expression values determined on the day prior to the challenge are shown in

Supplementary Figure 1. Of the 10 genes examined, only *Rora* and *Rev-erb* showed significant time-dependent expression differences prior to infusion ($P < 0.01$; one way ANOVA) with a peak at 06:00 h (Supplementary Figure 1).

The infusion of endotoxin triggered a significant decline in *Clock, Per3, Cry1, Cry2, Rora, CSNK1ε*, and *Rev-erb* expression, reaching the nadir within 3-6 hours (Figure 1A) ($P \leq 0.0001$ for all genes; one-way ANOVA). By 6 hours post-infusion, the expression values of these seven aforementioned genes had decreased by 80-90% relative to baseline. *Bmal1* expression was not altered significantly post-endotoxin infusion ($P > 0.05$, one-way ANOVA). The expression values of all genes, with the exception of *Bmal1*, differed significantly over the interval between 02:00 h (-7 h) pre-infusion and 02:00 h (17 h) post-infusion (Supplementary Table 1). These findings suggest that the expression levels of nine out of the ten genes examined remained suppressed for at least 17 hours post-endotoxin challenge.

Plasma cortisol and pro-inflammatory cytokines levels in endotoxin- and saline-challenged subjects

Plasma cortisol levels in humans normally peak 3-4 hours after the end of the sleep/darkness period [9,32]. Plasma concentrations of cortisol, as well as the pro-inflammatory cytokines TNFα and IL-6, increase significantly in response to an endotoxin challenge [22,31,33]. The anticipated increase in cortisol concentrations was detected in all endotoxin subjects by 1.5 hours post-challenge. The cortisol concentration peaked between 3-6 hours and returned to baseline levels at 24 hours (Figure 1C). As previously reported in this model system [22,33], TNFα concentration peaked within 1-1.5 hours post-infusion and returned to baseline by 3 hours (Figure 1D), whereas IL-6 concentration peaked within 2 hours and returned to baseline within 6 hours (Figure 1E).

Nocturnal study

We considered the circadian/diurnal phase at the time of endotoxin challenge as a possible influence on the expression of clock genes in endotoxin challenged PBL. We therefore also studied volunteer subjects challenged with endotoxin at 21:00 h (n=3). Consistent with the data presented in Figure 1A for the day subjects, the relative gene expression levels of all ten genes were close to or above baseline during the 24 hours pre-infusion (Figure 2). The infusion of endotoxin at 21:00 h triggered profound temporal changes in clock gene expression (Figure 2). The changes were similar to those seen in subjects challenged with endotoxin at 09:00 h. *Clock, Per3, Cry 1* and *Cry2, Rev-erb, Rora, and CSNK1ε* expression decreased by approximately 80-90% in response to the endotoxin challenge ($p < 0.001$) reaching their nadir between 4 and 9 hours post-infusion. As was observed in the day subjects, the nadir in *Per1* expression was reached several hours after the nadir of most other genes. *Per2* and *Bmal1* were the least affected by endotoxin. These observations establish that endotoxin suppresses clock gene expression when administered at various times during the diurnal cycle.

Circadian clock gene expression in PBL and leukocyte subpopulations

Circadian clock gene expression was also compared among PBL, monocyte- and neutrophil-subpopulations (n=3 for each). Blood was obtained prior to endotoxin-infusion (time 0; 09:00 h), and 6- and 24-hours post-infusion. As shown in Figure 3 (additional data are presented in Supplementary Figure 2), the expression patterns of *Clock, Cry 1-2, Rora, Per3, CSKN1ε, Rev-erb* and *Bmal1* noted in PBL were similar to those observed in neutrophils. The changes in *Clock, Cry1, Cry2, and CSKN1ε* expression observed in monocytes were time-delayed relative to those observed in neutrophils and PBL, whereas *Per3, Rora*, and *Rev-erb* expression were reduced in monocytes by 6 h post-infusion. Per1 expression showed a great degree of variability within cell populations even when derived from a single donor (Figure 3 and Supplementary Figure 2). In contrast, as observed in PBL, *Bmal1* expression was the least affected by

endotoxin in neutrophils and monocytes. The data suggest that the temporal changes in clock gene expression observed in PBL reflect changes that unfold in neutrophils, and to a lesser degree in monocytes.

Endotoxin does not affect the melatonin secretory rhythm

The plasma melatonin levels in humans normally peak during the night [34,35]. Analyses of plasma melatonin levels prior to infusion, and post-endotoxin or -saline infusion, revealed the anticipated increase in melatonin in the late part of the night (Figure 4). These observations suggest that endotoxin does not affect the apparent activity of the master clock during the acute period of systemic inflammation.

Circadian clock genes exhibit highly related responses to endotoxin

To better define the relationship among circadian clock gene expression in endotoxin-challenged PBL, the gene expression data presented in Figures 1 and 2 were clustered into a tree form (Supplementary Figure 3 A-D). Clustering analyses of the combined day- and night-placebo data suggested correlations between *Per2* and *Bmal1, and Cry1* and *Cry2* (Supplementary Figure 3 A). In contrast with the results observed for the placebo subjects, the clustering analyses identified four genes, *Clock, Per3, Cry1 and Cry2* that had highly related responses to endotoxin in both the day (Supplementary Figure 3 B) and night (Supplementary Figure 3 C) subjects. *Per 1, Per 2, Per 3*, and/or *Bmal1*, showed no correlated expression in subjects administered endotoxin (Supplementary Figure 3 B-D). Co-clustering of the day and night expression data indicated that *CSNK1ε* and *Rora* patterns correlate with *Clock, Per3, Cry1* and *Cry2* (Supplementary Figure 3 D). When the day and night endotoxin subjects data were analyzed as a group, the similar temporal decline in *Clock, Cry1, Cry2, Per3, CSNK1ε*, and *Rora* expression became apparent (Figure 5). The line featured in each of the panels in Figure 5 represents the mean of *Clock, Cry1, Cry2* and *Per3* expression values. *Bmal1, Rev-erb, Per1*, and *Per2* displayed distinct expression patterns as compared to the common response pattern of the remaining six genes. The large number of clock genes exhibiting a similar endotoxin-induced response suggests that clock gene expression is both synchronized and suppressed during the acute period of systemic inflammation.

Discussion

The expression of many circadian clock genes is suppressed in PBL during the acute period of systemic inflammation

We found that the expression of key genes implicated in the regulation of circadian clock function, including *Clock, Per3, Cry1* and *Cry2, Rora*, and *Rev-erb*, is decreased in PBL by 80-90% within 3-6 hours post-endotoxin infusion. The *Clock/Bmal1* complex regulates the transcription of genes with an E-box promoter region, which include *Period (Per1, Per2, Per3)*, *Cryptochrome (Cry1, Cry2)* and *Rev-erb* [36]. Per/Cry protein complexes relocate from the cytosol to the nucleus, where they function as *Clock/Bmal1* repressors, while *Rev-erba* and *Rora* bind to the promoter region of *Bmal1* to either suppress or enhance its expression, respectively [3]. The repressor Per proteins are phosphorylated by Casein kinase 1ε (CSNK 1ε) [37]. The phosphorylated proteins are ubiquitinated and targeted for degradation by the proteosome. Once the Per proteins are degraded, the de-repressed *Clock/Bmal* complex reinitiates its activity cycle. The precipitous declines in expression of multiple genes that act at various points in the circadian clock network noted in this study provide a strong indication that clock activity in PBL is severely impaired during the acute period of systemic inflammation.

Per1, Per2, Per3, and *Bmal1* gene oscillations have been observed in human peripheral blood mononucleated cells (PBMCs) as well as whole blood cells [9,32,38,39]. In one study [32],

Per1 and *Per2* expression in PBMCs peaked in the early hours of the day, whereas *Bmal1* peaked in the middle of the wake period. In another study, *Per2* and *Bmal1* cycled with a similar rhythm in PBMCs [39]. Recently, the expression of ten circadian clock genes, including *Per 1-3*, *Cry1* and *Cry2*, *Clock*, and *Bmal1*, was examined in PBMCs [35]. Of the ten genes examined, only *Per 1-3* showed rhythmic expression in most subjects, with no significant acrophase differences among the three genes. The goal of the present study was to determine the fate of clock gene expression in PBL during the acute period of systemic inflammation. Hence, in contrast with the entrainment protocols used by others [9,32,35,39], our subjects were not entrained by an extended sleep/wake schedule prior to or during the study phase. These differences may explain the limited similarity among *Per* gene expression patterns noted among our placebo subjects.

Prior studies revealed a limited coordination among clock gene expression patterns in PBMCs obtained from sleep/wake entrained normal human subjects, and a significant inter-subjects variability [32,35,39]. In contrast, *Cry1* and *Cry2*, as well as *Clock*, *Per3*, *CSNK1ε*, *Rev-erb* and *Rora* exhibited correlated expression in PBL obtained from endotoxin subjects. The correlated expression patterns observed in PBL, neutrophils and monocytes after an endotoxin challenge suggest that endotoxin is a potent entrainer of the circadian clock network in circulating inflammatory cells.

Melatonin, cortisol, and circadian clock gene expression in PBL during the acute period of systemic inflammation

In humans, the activity of the central master clock is generally correlated with the secretion of melatonin in the middle to late part of the night [40] [34,35,39]. We found that the melatonin rhythms remained intact in endotoxin-challenged subjects, peaking in the late part of the night. As previously reported [31], endotoxin triggered a surge in plasma cortisol levels with a peak between 3-4 hours. While cortisol levels peaked, many clock genes reached their expression nadir. These data suggest that centrally regulated plasma melatonin- and cortisol-rhythms, and PBL clock gene expression are independently regulated in response to endotoxin. These observations raise the possibility that the master clock and circadian clock gene expression in PBL become misaligned during the acute period of systemic inflammation induced by endotoxin.

Okada et al [21] recently observed transient changes in clock gene expression in SCN and liver of endotoxin challenged rats. In that study, endotoxin did not affect the rhythmicity of clock gene expression. Consistent these observations, our data also indicate that endotoxin transiently suppresses clock gene expression in peripheral tissue(s). Furthermore, our data establish that significant perturbations in clock gene expression in PBL unfold while the rhytmicity of the master clock, determined based on plasma melatonin levels, appears to remain intact in humans.

Despite our incomplete understanding of the mechanism(s) by which endotoxin influences circadian rhytmicity, a limited comparison between endotoxin and the two other well-characterized circadian entrainers, light and food, is possible. A short and abrupt period of bright light during the dark period is sufficient to reset the activity of the central clock in humans, as determined by changes in core body temperature and plasma cortisol production [41]. The magnitude of light-induced circadian phase resetting is dependent on the phase of the clock at the time of the input [41]. In contrast, endotoxin appears to trigger similar changes in circadian gene expression in PBL irrespective of the central clock phase. In addition, endotoxin disrupts the temporal relationship among plasma cortisol and melatonin, and circadian gene expression in PBL. These observations suggest that the mechanism by which systemic endotoxin affects clock gene expression differs from the process by which light entrains the clock. The effect of endotoxin on the circadian activity may be more similar to that of feeding. In rodents, feeding during the subjective night or the subjective day alters the

rhythmic expression of *Per1*, *Per2*, *Per3*, and *Cry1*, in the liver within a few days. In contrast, the phase of *Per1* and *Per2* expression in the SCN was not affected by food entrainment [42]. Thus, both endotoxin and feeding appear to affect peripheral clock gene expression but not the activity of the central master clock.

Pro-inflammatory cytokines and clock gene expression in PBL

Pro-inflammatory cytokines, which are released in the early stages of systemic inflammation, have been implicated in the regulation of circadian activity in mice. TNFα- or IL-1β-infusion suppressed the expression of several clock genes, including *Per2* and *Per3*, in mice livers by binding to the E-box motifs in their promoters [20]. *Clock* and *Bmal1* expression was not affected these cytokines [20]. TNFα levels surge in response to endotoxin, reaching a zenith within 1.5-2 hours post-challenge, while the expression of most clock genes examined in this study remained suppressed for up to seventeen hours. Thus, while TNFα and/or IL-1β are likely to contribute to the regulation of several clock genes, additional factors appear to regulate clock gene expression in human PBL during the acute period of systemic inflammation.

Boivin et al. [9] were the first to examine circadian clock gene expression in human PBMCs. Analysis of PBL circumvents the need for cell-purification step(s), minimizes manipulation time, and hence is a practical approach for sample acquisition in the clinical setting. However, the use of a mixed cell population such as PBL introduces some level of uncertainty as the proportion of each immune cell type changes dynamically after endotoxin challenge, returning to baseline within 12 hours post-treatment [22,43,44]. To address this potential limitation, clock gene expression was compared among PBL, monocytes, and neutrophils at select time points post-infusion. The studies revealed that the changes in clock gene expression observed in PBL were replicated in neutrophils. Interestingly, by 6 hours post-endotoxin infusion, several but not all clock genes, were also suppressed in monocytes. These findings establish that the changes in circadian clock gene expression observed in PBL are primarily reflective of changes occurring in neutrophils, and to a lesser extent in monocytes.

In conclusion, our study defines a severe misalignment of central circadian entrainment cues from peripheral clock gene expression in PBL during acute systemic inflammation. Godin and Buchman [45] proposed that systemic inflammatory responses might trigger uncoupling between biological oscillators. Others have reported that the circadian rhytmicity of melatonin secretion is suppressed in severely ill patients [46,47], while in rodents, absence of circadian cues during recovery from sepsis impairs survival [48]. These findings add a new element to the potential link between circadian regulation and disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

IL	interleukin
PBL	peripheral blood leukocytes

PBMCs	peripheral blood mononucleated cells
SCN	suprachiasmatic nucleus
TNF	tumor necrosis factor

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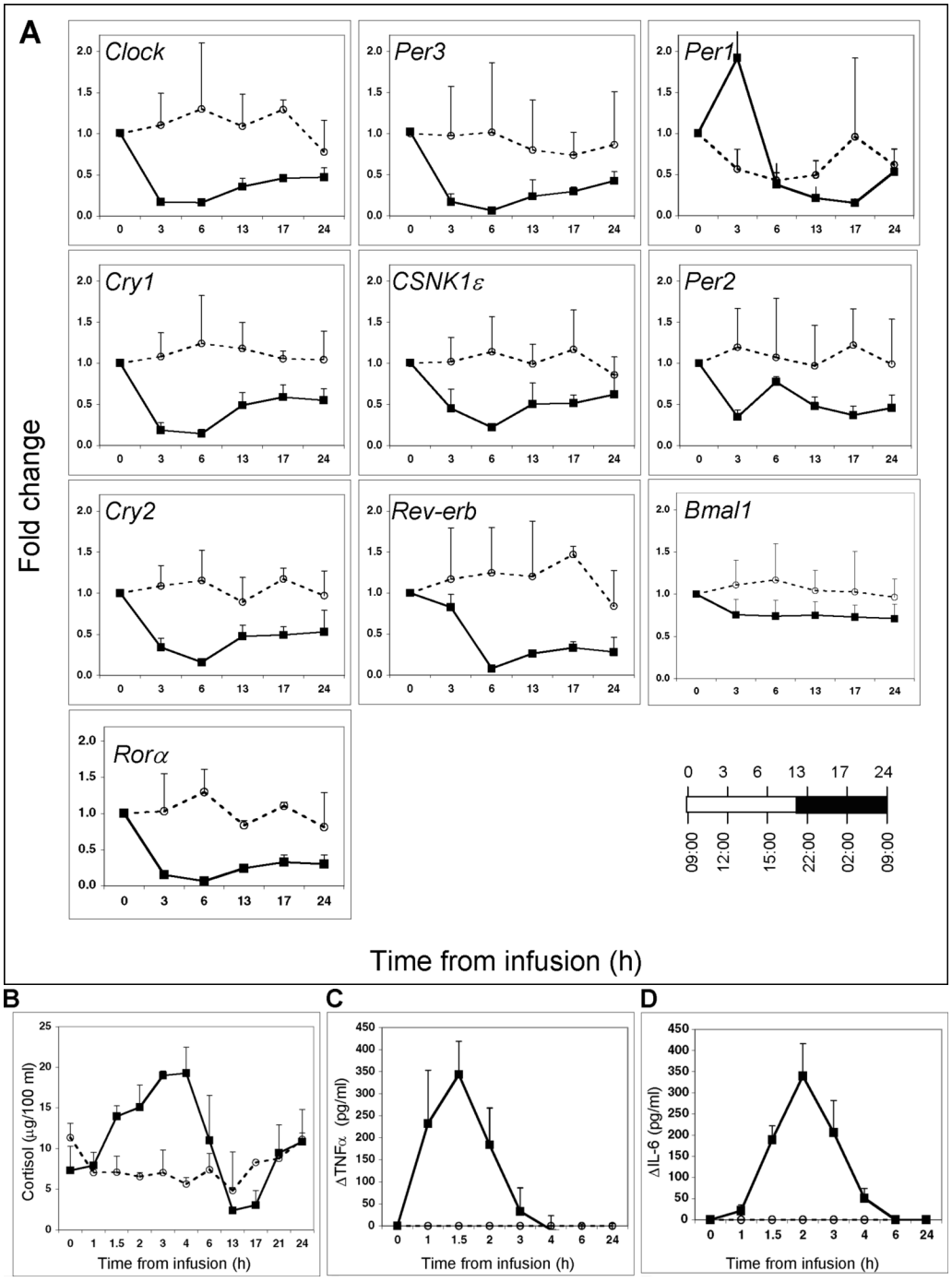

Figure 1. Clock gene expression in peripheral blood leukocytes obtained from human subjects challenged with endotoxin or saline at 09:00 h

(A) Blood was collected at the indicated time points post-endotoxin (closed symbols; n=4 subjects) or post-saline (open symbols; n=2 subjects) infusion. Gene expression levels were analyzed by qRT-PCR and are expressed as fold change relative to the 09:00 h infusion time, taken to be time 0. The relationship between the time from infusion and the time-of-day is illustrated in the lower right hand corner. Data are expressed as mean fold change ± SD. (B) Plasma cortisol levels (n=4) as a function of time post-endotoxin were monitored by direct

radioimmunoassay. (C) TNFα (n=4) and (D) IL-6 (n=4) levels were determined by enzyme-linked immunoassay.

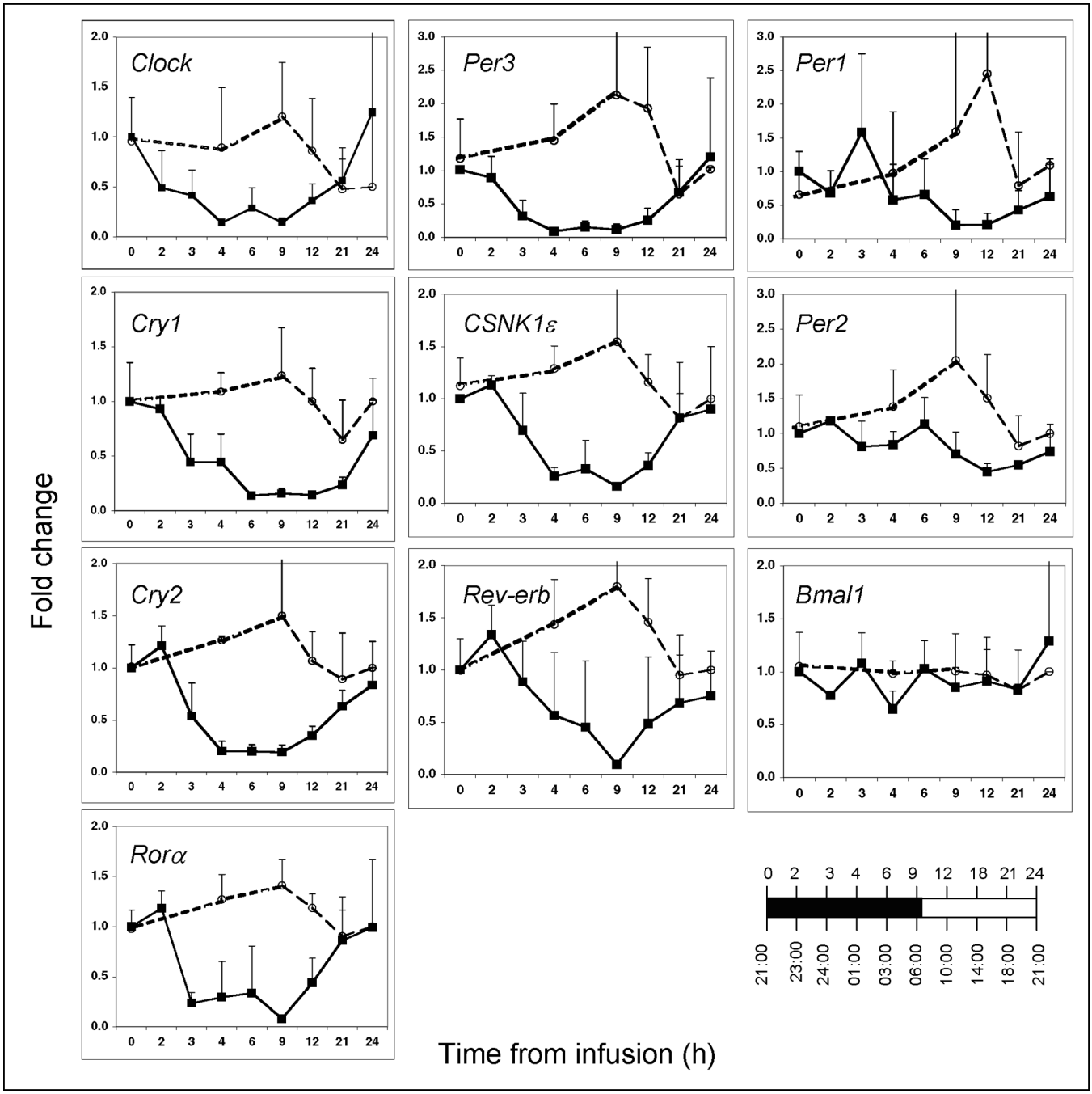

Figure 2. Clock gene expression in peripheral blood leukocytes obtained from human subjects challenged with endotoxin at 21:00 h

Blood was collected at the indicated times prior to (open symbols) and post-endotoxin infusion (closed symbols) (n=3 subjects). Gene expression levels were analyzed by qRT-PCR and are expressed as fold change relative to the 21:00 h infusion time, taken to be time 0. The relationship between the time from infusion and the time-of-day is illustrated in the lower right hand corner.

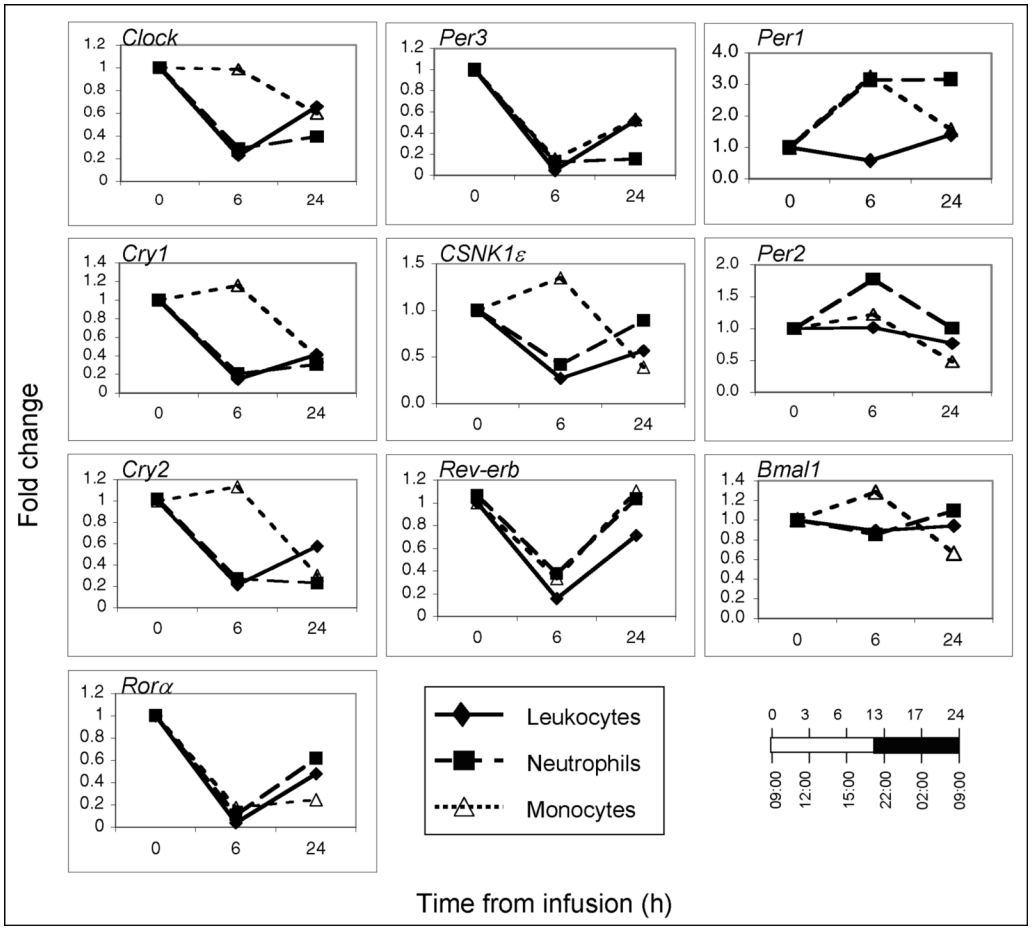

Figure 3. Clock gene expression in peripheral blood leukocytes, neutrophils and monocytes obtained from human subjects challenged with endotoxin at 09:00 h

Blood was collected at time 0, 6 and 24 hours post-endotoxin infusion. Total peripheral blood leukocytes, neutrophils and monocytes subpopulations (n=3 for each) were obtained prior to RNA isolation. Gene expression levels were analyzed by qRT-PCR and are expressed as fold change relative to the 09:00 h infusion time, taken to be time 0. The relationship between the time from infusion and the time-of-day is illustrated in the lower right hand corner. The data shown are averages (n=3) (all data sets are presented in Fig. 2S).

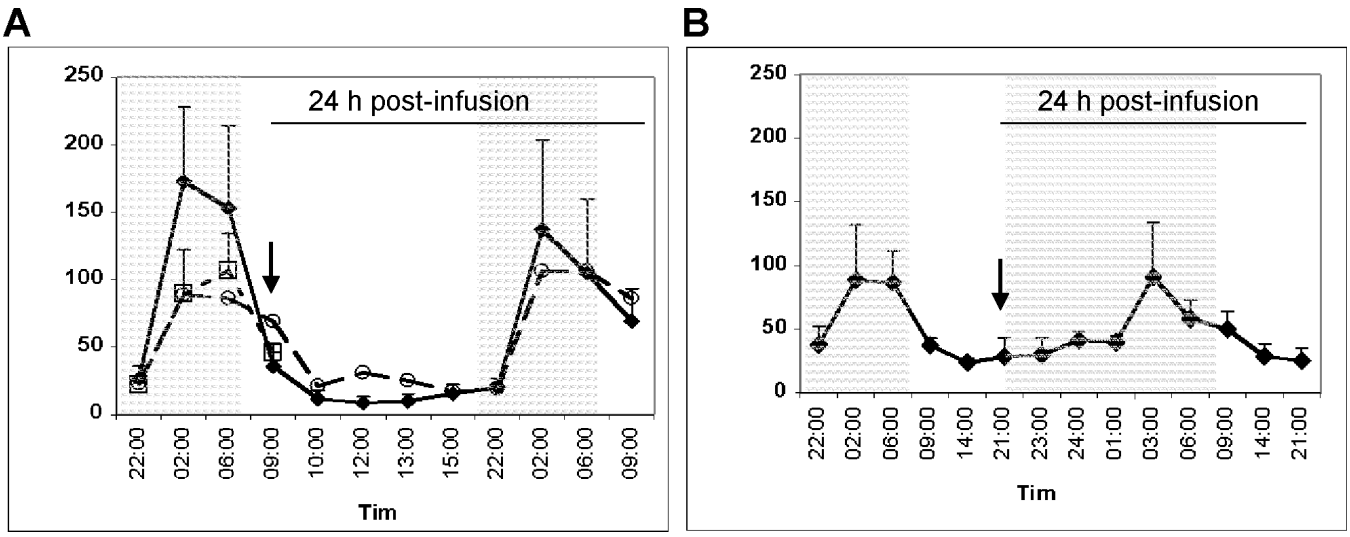

Figure 4. Endotoxin does not alter the melatonin secretory rhythm

(A) Plasma melatonin concentrations were determined in blood samples obtained from control subjects (n=8 subjects) at 22:00 h, 02:00 h, 06:00 h, 09:00 h and 10:00 h (open squares). Samples were also obtained at the indicated times of the day pre- and post-saline infusion (open circles; n=2 subjects), and pre- and post-endotoxin infusion (closed diamonds; n=4 subjects). Arrow indicates the 09:00 h infusion time. (B) Samples were obtained at the indicated times of the day pre- and post-endotoxin infusion (closed diamonds; n=4 subjects). Arrow indicates the 21:00 h infusion time.

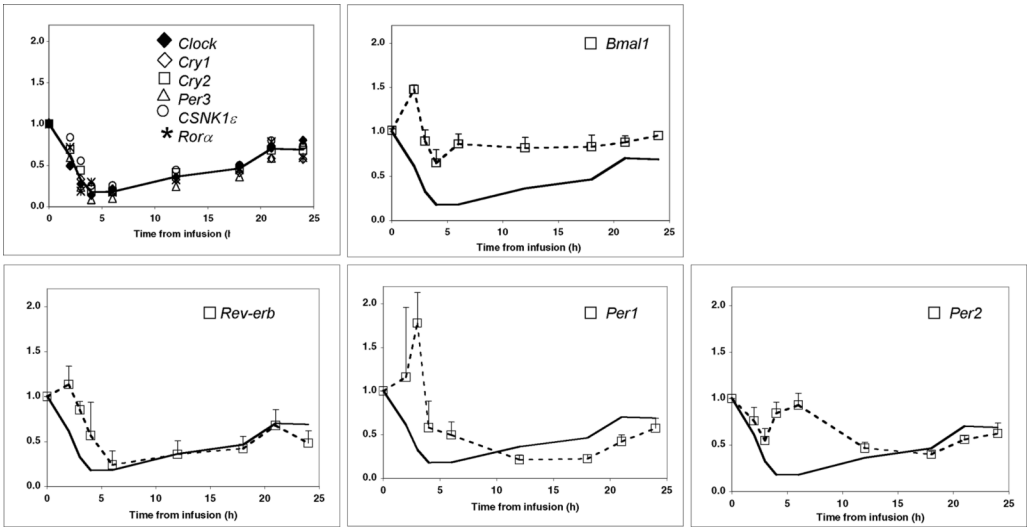

Figure 5. Clock gene expression in human peripheral blood leukocytes

The relative gene expression values for each gene post-endotoxin infusion were combined for all day (n=4 subjects) and night subjects (n=3 subjects), and are presented as an average. Where shown, bars represent standard error of the mean. The line that is featured in each panel was drawn based on the mean expression values of *Clock*, *Cry1*, *Cry2*, and *Per3*, which exhibited correlated expression in both the day and night subjects.