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Circadian responses to endotoxin treatment in mice

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Abstract

We tested the ability of *Escherichia coli* lipopolysaccharide (LPS) to phase-shift the activity circadian rhythm in C57Bl/6J mice. Intraperitoneal administration of 25 μg/kg LPS induced photic-like phase delays (−43±10 min) during the early subjective night. These delays were non-additive to those induced by light at CT 15, and were reduced by the previous administration of sulfasalazine, a NF-κB activation inhibitor. At CT 15, LPS induced c-Fos expression in the dorsal area of the suprachiasmatic nuclei (SCN). Our results suggest that the activation of the immune system should be considered an entraining signal for the murine circadian clock.

Keywords: LPS; phase response curve; c-Fos; sulfasalazine; NF-κB

1. Introduction

Circadian rhythms are generated by endogenous pacemakers and thus persist in the absence of external periodic signals. The suprachiasmatic nuclei (SCN), located in the anterior hypothalamus, are considered to be the main circadian clock in mammals (Klein et al., 1991; Reppert and Weaver, 2002), although other "peripheral" oscillators have been located in diverse tissues (Balsalobre et al., 2000; Tosini and Menaker, 1996; Yoo et al., 2004). In order to generate precise 24 h rhythms, these nuclei are entrained to environmental signals, notably the light–dark cycle, by daily adjustments in the phase of the oscillations. Light pulses administered under constant dark conditions affect the clock differentially depending on the time of stimulation: early night pulses delay and late night pulses advance the phase of the oscillation, while diurnal light pulses do not affect the clock, defining what is known as a photic phase-response curve (PRC) (Meijer and Schwartz, 2003). The SCN drive a number of behavioral and physiological rhythms, such as locomotor activity, food intake, body temperature and several endocrine secretions.

Circadian oscillations of hormones and other signals (including immune factors), as well as behavioral changes have been established during physiological and pathological states in humans and in laboratory animals. Several studies have demonstrated daily and circadian (near 24 h) rhythms in circulating levels and functions of lymphoid cells and their subsets (Born et al., 1997). Nevertheless, the study of the rhythmicity of the immune system has focused mainly on its pathological aspects and few studies have tried to address its integrative role from a physiological point of view.

Although there is a substantial amount of information regarding the circadian modulation of many immunological variables (Floyd and Krueger, 1997), there are almost no data about the possible effect of immune factors on the circadian system itself. However, several reports suggest a possible immune feedback regulation of the circadian clock. The sleep–wake cycle (one of the most evident circadian rhythms) is modified by pro- and anti-inflammatory

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cytokines (Krueger et al., 2001) and immunosuppressant drugs such as cyclosporin A and tacrolimus affect the phase of locomotor activity rhythms in laboratory animals (Golombek et al., 1998). Moreover, immune-related transcription factors are present and active in the SCN and its activity is partially necessary for light-induced phase shifts (Marpegan et al., 2004).

Introduction of gram-negative bacteria into the body causes the liberation of toxic, soluble products of the bacterial cell wall, such as lipopolysaccharide (LPS, also known as endotoxin). The administration of LPS has been used to evaluate its somnogenic activity (Mullington et al., 2000), as well as its effect on memory consolidation and on behavior (Gahtan and Overmier, 2001; Pugh et al., 1998). It is now well established that LPS induces autonomic, endocrine and behavioral responses that are controlled by the brain (Linthorst and Reul, 1998; Matsunaga et al., 2000). Peripheral administration of LPS exerts profound effects on the sleep–wake cycle and sleep architecture and may produce, at higher doses, fever and a characteristic "sickness behavior" observed during inflammatory diseases, including sleep pattern changes and fever oscillations along the day (Kluger, 1991; Krueger et al., 1998). In mice, susceptibility to lethal doses of endotoxin increase dramatically during the resting period (Halberg et al., 1960) and a similar temporal pattern of induced mortality has also been established for tumor necrosis factor α (TNFα) (Hrushesky et al., 1994).

In this context, we hypothesized that low doses of LPS, known to cause an increase in proinflammatory cytokines (i.e. IL-1, TNFα and IL-6), would produce synchronizing effects on the circadian system. We report here that LPS induce changes in the phase of locomotor activity rhythms in a manner similar to light-induced phase delays, but do not have an additive effect when co-administered with a light pulse. The LPS treatment stimulated the dorsal area of the SCN as assessed by c-Fos activation. The phase-shifting response to LPS was reduced when the activation of NF-κB, a transcription factor reported previously to play a role in the photic input of the circadian system (Marpegan et al., 2004), was prevented by sulfasalazine.

2. Experimental procedures

2.1. Animals

Adult C57-BL/6J male mice (*Mus musculus*) were raised in our colony, housed under a 12:12 light/dark photoperiod or constant dark conditions, with food and water ad libitum. For the experiments in constant darkness, mice were transferred to constant dark conditions in single cages 20 days prior to the treatments. When needed, mice were sacrificed by a lethal dose of a ketamine/xylazine cocktail at the end of the experiments. All animals were treated only once and all drugs were delivered in a single administration. All efforts were made to minimize the number of animals used and their suffering. All animal experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals. LPS from *Escherichia coli* serotype 0111:B4 was purchased from Sigma (St. Louis, MO). Unless stated otherwise, all drugs came from Sigma.

2.2. Immunohistochemistry

Mice were deeply anaesthetized with a cocktail containing ketamine (150 mg/kg) and xylazine (10 mg/kg) 90 min after LPS, saline or light treatment at *zeitgeber* time (ZT) 15 (with ZT 12 defined as the time of lights off) and perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were carefully removed, post-fixed overnight, cryoprotected in sucrose 30% in 0.01 M PBS for 24 h and 25 μm thick coronal sections were cut with a cryostat and collected in 0.01 M PBS. Free floating slices were blocked in 5% nonimmune horse serum (NHS) and incubated with primary antisera raised in rabbit against c-Fos (Santa Cruz Biotechnology, 1:2500 dilution in 2% NHS containing 0.1% Triton X-100) for 24 h at 4 °C. Sections were then treated using the avidin–biotin method with a Vectastain Elite Universal kit containing a biotinylated universal secondary antibody (Vector Laboratories, Burlingame, CA), with diaminobenzidine/cobalt as a chromogen.

2.3. Behavioral analysis

The wheel-running circadian rhythm of mice was recorded with a Dataquest III acquisition system (Minimitter, Sunriver, OR). Wheel revolutions were monitored by magnetic microswitches activated by the wheel and collected every 5 min. The onset of activity was defined as CT 12 and was used as a phase reference point to calculate phase shifts. In order to construct an LPS induced phase-response curve (PRC), mice were treated at different circadian times (CT 3, CT 7, CT 11, CT 15, CT 19 and CT 23, n=4–8, per treatment and group) with a 25 μg/kg 100 μl intraperitoneal injection of LPS from *E. coli* serotype 0111:B4 Sigma or an equal volume of saline. The responses to lower (5 μg/kg) and higher (250 μg/kg) LPS doses were analyzed at CT 15 (n=4 for each dose), the time when light pulses induce phase delays of the free-running rhythm. The interaction of LPS treatment with light pulses was also studied at CT 15 administering 25 μg/kg of LPS 10 min prior to a nonsaturating 5-min 50-lx light pulse or to a saturating 10 min 400 lx pulse. In order to block the LPS-induced phase shift, sulfasalazine (Sigma) diluted in 15% DMSO (Fluka Chemie, Buchs) in saline, a potent and specific NF-κB activation inhibitor was administered intraperitoneally at a 100 mg/kg dose 1 h prior to a 25 μg/kg LPS injection (n=9). A control group was injected with sulfasalazine 1 h prior to saline treatment at CT 15 and a second control group was treated with vehicle (15% DMSO in saline) at CT 14 and LPS at CT15 (n=4). In all the

experiments phase shift responses were analyzed with onset data from days 4–14 after treatment (discarding the data from the 3 days post-stimulation), assigning positive values to phase advances and negative values to phase delays.

2.4. Temperature analysis

Temperature sensors (VM-FH model, Mini-mitter) were implanted i.p. into deeply anesthetized mice and the corresponding receivers (RA-1000) were located under the animal cages. Eight days after surgery the animals that showed normal temperature rhythms were divided into three groups, which received LPS (25 or 250 μg/kg) or saline at CT 15. Body temperature was recorded every 5 min before and after treatment.

2.5. Statistical analysis

Phase shifts were estimated by three independent observers blind to the experimental procedure. The data obtained in the phase response curve and the co-administration experiments was analyzed by two-way ANOVA. One-way ANOVA was used to analyze the rest of the experimental data (Bonferroni and Dunnett post tests were used in the dose curve and the sulfasalazine experiments, respectively). For the Dunnett test, LPS was considered as the control treatment. Data are presented as mean±S.E.M. *P* values of 0.05 or less were considered to be statistically significant.

3. Results

3.1. Phase response curve to LPS

Running-wheel locomotor activity rhythms were analyzed in order to study the phase shifts induced by the administration of subpyrogenic doses of LPS. A phase response curve (PRC) was constructed by plotting the phase shift induced by different treatments (100 μl 25 μg/kg LPS or 100 μl saline solution, i.p.) against injection time. Mice kept under constant darkness were treated at 4-h intervals including circadian times (CT) 3, 7, 11, 15, 19 and 23 (n=4–8 per treatment and time point). When a 25 μg/kg dose of LPS was administered at CT 15 (3 h after activity onset), a phase delay of −43±10 min was observed (p<0.01 for treatment variable, p<0.001 for time variable and p<0.001 for interaction, treatment x time, two-way ANOVA), while no significant phase shifts occurred when animals were injected at other circadian times (see Fig. 1A).

LPS dose effects were also tested at circadian time 15. Mice were injected with three different LPS doses (5, 25 or 250 μg/kg) and phase responses were measured. Animals treated with 25 and 250 μg/kg LPS injections showed phase delays significantly different from saline injections (Fig. 1A, inset; p<0,001, one-way ANOVA) but not statistically different between them (p>0.05, Bonferroni post test between 25 and 250 μg/kg), while a 5 μg/kg dose did not phase shift locomotor activity rhythms.

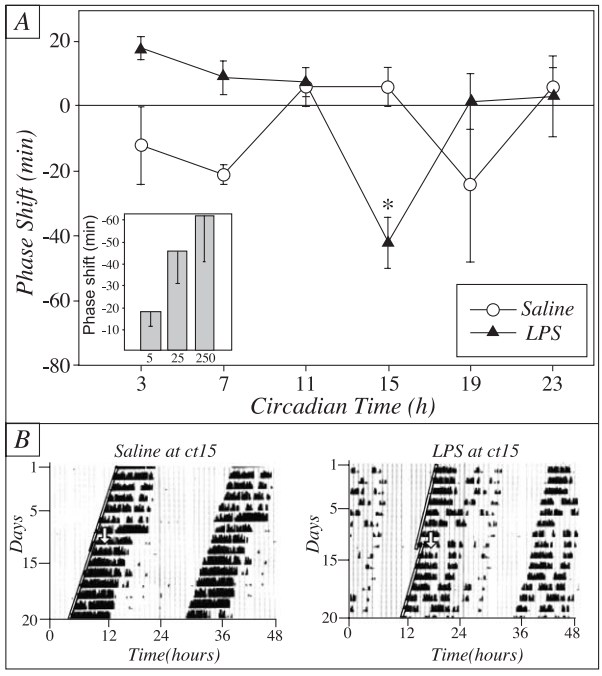

Fig. 1. LPS-induced phase response curve (PRC). I.p. LPS treatment (25 μg/kg) induced significant phase delays when injected at CT 15 (p<0.01, ANOVA) but had no significant effect when animals were treated at other circadian times (CT 3, CT7, CT11, CT 19 and CT 23). CT 12 was defined as the activity onset and used as reference phase for each animal. Negative values represent delays and positive values indicate phase advances. Circles represent the phase shifts induced by saline treatment and black triangles represent the response to LPS (n=4–8 for each treatment and time point). Inset: Three different LPS doses were injected i.p. at CT 15. No phase shift response was observed with the lower dose (5 μg/kg), while a 250 μg/kg dose induced larger, although non-statistically different, responses than the 25 μg/kg dose. (B) Representative double-plotted actograms of animals treated with saline or LPS at CT 15. Treatments are indicated by white arrows on the actograms.

3.2. Endotoxin effects on temperature and behavior

Since temperature changes might be an input to the circadian system and mask the humoral-induced phase shifts, we studied the effect of the 25 and 250 μg/kg LPS injections on core body temperature. There were no temperature changes in mice treated with saline or with a 25 μg/kg dose while, as reported by other groups (Blanque et al., 1999; Dogan et al., 2000; Paul et al., 1999), a hypothermic effect that lasted for about 4 h was induced by the higher LPS dose (data not shown). The hypothermic effect caused by the 250 μg/kg LPS administration, evident 1 h after the injection, produced a decrease in the core body temperature of −3±0.5 °C and was markedly different from the effect of the 25 μg/kg dose (p<0.01, one-way ANOVA). When a light pulse was given at CT15, animals did not

show temperature variations different from those observed in saline treated animals (data not shown).

In order to study the appearance of sickness behavior, an ethogram for each treatment was performed. The group treated with the higher LPS dose showed a clear and long-lasting sickness behavior that included shivering and stillness while animals treated with the lower dose showed a milder and shorter response that included stillness but not shivering. LPS or light pulses instantly produced an increased rate of sniffing and rearing, with a temporary decrease in total and wheel-running locomotor activity. The animals treated with 250 μg/kg LPS experienced shivering and stillness that lasted for about 10 h more than the 25μg/kg treated group. The clear evidence of core body temperature changes and sickness behavior in animals treated with 250 μg/kg LPS prevented us from using this dose in the next experiments since core body temperature changes adds an undesirable confounding effect.

3.3. Effects of LPS and light pulse co-administration on circadian phase

Since LPS administration induced a photic-like PRC, the interaction between both stimuli (light and LPS) was studied in order to find whether they share a common pathway or if they are independent from each other. Administration of a light pulse at the early subjective night (activity phase) in rodents induces phase delays that depend on the light pulse intensity and duration. A non-saturating 5 min light pulse (50 lx) administered at CT 15 produced a −39±5 min phase delay of the locomotor activity rhythm, while a 15-min saturating light pulse (400 lx) produced a −126±24 min phase delay. A 25 μg/kg LPS i.p. injection at CT15 induced a phase delay of −38±7 min (see Fig. 2) while the co-administration of

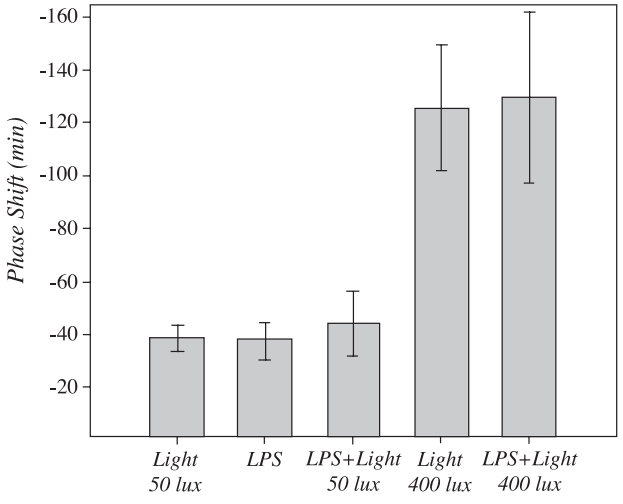

Fig. 2. Co-administration of light pulses and LPS shows no additive phase shift responses. Saturating (10 min, 400 lx) or non-saturating (5 min, 50 lx) light pulses were applied at CT 15 together with a 25 μg/kg i.p. dose of LPS or a control saline solution. No significant differences between light and light+LPS treatments were found in the phase shift responses.

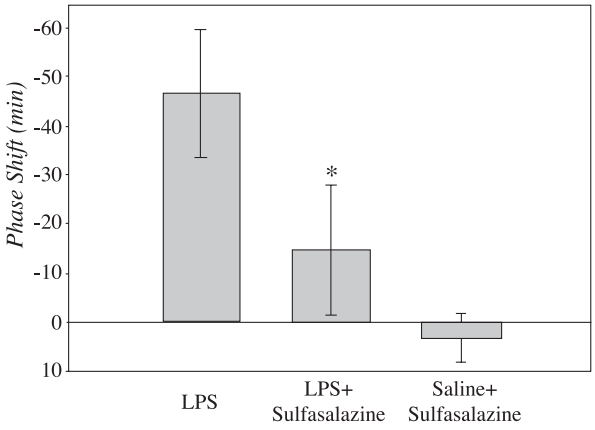

Fig. 3. Sulfasalazine (100 mg/kg) reduces the phase shift response to LPS. Sulfasalazine (100 mg/kg) injected i.p. 1 h prior to a 25 μg/kg LPS injection blocked the LPS induced phase shift response ($p<0.01$ vs. LPS, Dunnett post test). LPS: vehicle injection 1 h before LPS treatment, LPS+Sulfasalazine: sulfasalazine treatment prior to LPS administration, Sulfasalazine: sulfasalazine treatment.

LPS and a non-saturating light pulse rendered a −44±12 min phase delay. When the co-administration included a saturating light pulse, a −130±32 min phase delay was observed. The statistical analysis of this data rendered a significant effect for light ($p<0.0001$) and LPS treatments ($p<0.03$) and a non-significant effect for LPS-light interaction ($p>0.05$). This may be interpreted as LPS being ineffective to modify the phase shifts induced by light. In other words, no additive effects were obtained when LPS and light pulses (in saturating and non-saturating conditions) were co-administered.

3.4. Effect of sulfasalazine on LPS-induced phase shifts

Immune suppressors that inhibit NF-κB activity block the classical immune-mediated LPS response. Therefore, sulfasalazine, a drug that prevents the disengagement of the inhibitor protein IκB from NF-κB (Wahl et al., 1998) was used to analyze its effect on LPS-induced phase delays.

The phase delay produced by 25 μg/kg of LPS at CT 15 (−47±13 min) was significantly reduced by a 100 mg/kg sulfasalazine i.p. injection 1 h prior to the 25 μg/kg LPS treatment (−15±13 min, $p<0.01$ vs. LPS treatment, one-way ANOVA followed by Dunnett's test). The administration of 100 mg/kg sulfasalazine plus saline did not affect circadian phase (3±5 min) (Fig. 3).

3.5. LPS and light pulse effects on c-fos induction on the circadian system

Light pulses applied at CT 15 induce c-*fos* expression in the mouse SCN (Castel et al., 1997; Colwell and Foster, 1992; Yamazaki et al., 2002). In order to study if LPS treatment is capable of producing the same response than light in terms of c-*fos* expression, immunohistochemistry against the c-Fos protein was carried in SCN-containing

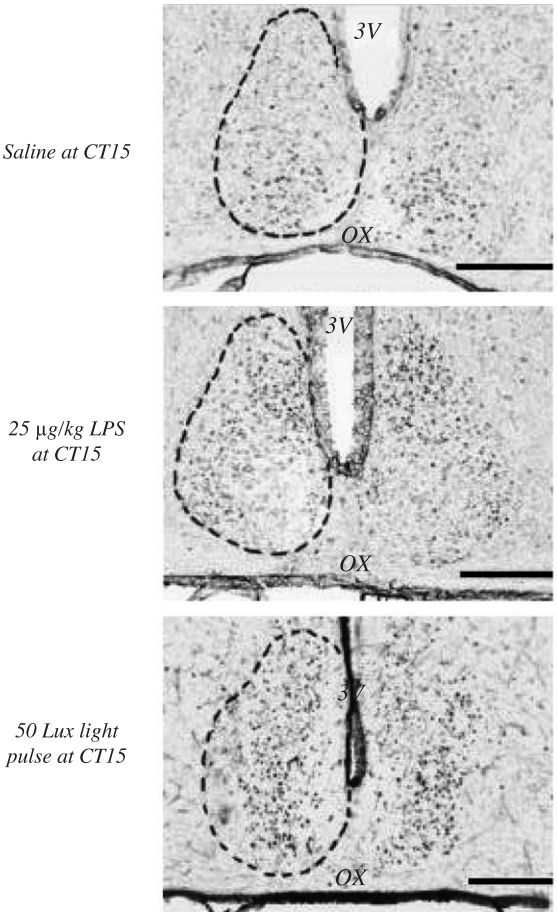

Fig. 4. Fos expression in the SCN is induced by LPS or light. c-Fos immunocytochemistry was performed in brains from animals treated at CT 15 with 25 μg/kg LPS, saline or light pulses (5 min, 50 lx). Three representative sections are shown, exhibiting the differential patterns of expression in the SCN. Immunoreactive cells were distributed all along the SCN in the light-treated animals, while a dorsal distribution of the immunoreactivity was evident in the LPS treated mice. OX, optic chiasm; 3V, third ventricle; bar, 200 μm.

coronal brain slices obtained from animals treated with light pulses, LPS or saline at CT 15.

As expected, mild immunoreactivity was observed after saline treatment while a strong expression of c-Fos through all the SCN was induced by light pulses. LPS administration induced a different pattern of c-Fos expression, predominantly in the dorsal portion of the SCN (Fig. 4). A strong immunoreactive signal was also evident in the paraventricular nucleus of the hypothalamus (PVN) after LPS treatment.

4. Discussion

In the present work we analyzed the circadian response to intraperitoneal injections of bacterial lipopolysaccharide in mice. Our results show clearly that the administration of LPS at a subfebrile dose produces photic-like phase delays at the beginning of the subjective night. This phase delaying effect of LPS was not additive with photic stimuli, and was blocked by the previous administration of sulfasalazine, a specific NF-κB inhibitor. While light pulses induced c-*fos* activation through all the SCN, LPS treatment induced c-*fos* was restricted to the dorsal region. Altogether, these data present the first in vivo evidence of the response of the circadian system to an immune challenge such as LPS and, since we used relatively low doses of endotoxin, suggest the existence of a feedback pathway from immune effectors into the circadian system.

As can be seen in Fig. 1, the i.p. administration of 25 μg/kg of *E. coli*-derived LPS caused a significant phase delay at CT15 but had no significant effect when injected at other circadian times (CT 3, 7, 11,19 and 23). The phase delays of the curve resembles those induced by light stimulation in C57BL/6J mice (Schwartz and Zimmerman, 1990), so we conclude that LPS induces photic-like phase delays.

Administration of LPS induces a complex response that may include the activation of the immune, endocrine and nervous systems. This response may differ according to the dose, time of injection and the general state and history of the animal. The alteration of core body temperature (hyper and hypothermia) is one of the most classic and studied responses to an LPS challenge. The alterations in core body temperature and rectal temperature in response to LPS challenges is not clearly dose-dependent and may include fever, hypothermia or alternation of both responses (Blanque et al., 1999; Dogan et al., 2000; Paul et al., 1999). Nevertheless, the phase delays observed in response to LPS were not caused by an alteration in body temperature, since no hyper- or hypothermia was seen after the animals were injected with the 25 μg/kg dose. Low dose administration of LPS did have some minor behavioral effects; however, it is unlikely that these subtle changes mediate phase shifting. With higher doses, however, LPS exerts profound effects on behavior, including hypoactivity and other thermoregulatory changes, which are also time-dependent (Mathias et al., 2000).

One of the pathways through which LPS could affect the circadian system to induce phase shifts might be an increase in adrenal secretions, since its i.p. administration results in an increase of plasma corticoids (Lenczowski et al., 1998). However, it has been reported that glucocorticoids may synchronize peripheral oscillators but do not affect the phase of SCN neurons, probably due to the absence of specific receptors in the SCN (Balsalobre et al., 2000). Hence, the phase delay induced by LPS is unlikely to be mediated by the hypothalamic–pituitary–adrenal axis (HPA) activation.

The mechanism by which peripheral administration of LPS produces central nervous system responses remains controversial. It has been shown that LPS (100 μg/kg) is not capable to cross the brain–blood barrier in rats but it can bind to specific receptors in cerebral vascular endothelium inducing proinflammatory responses (Singh and Jiang, 2004). Cytokines induced by LPS may also enter into the brain through the organum vasculosum laminae terminalis

where the blood–brain barrier is "leaky" due to the presence of a fenestrated endothelium (Romanovsky et al., 2003) or act through neural terminals of peripheral tissue, e.g., those of the vagus nerve (Maier et al., 1998; Simons et al., 1998). Further work should be done in order to determine the relevance of each of these pathways in the circadian-immune cross talk.

The fact that LPS and light co-administration at CT 15 showed no additive effects suggests that there is some interaction between both pathways. We have recently demonstrated that light-induced phase advances could be blocked by the NF-κB pathway inhibitor pyrrolidinedithiocarbamate in hamsters (Marpegan et al., 2004). Therefore, we can hypothesize that NF-κB activity, or components of its activation pathway (including κB-activated genes) might act as convergence points between photic and LPS circadian effects. This is in accordance with our present finding that sulfasalazine block LPS-induced phase delays in mice. This inhibition might be caused by the suppression, within the hypothalamus or in the periphery, of the LPS-induced release of proinflammatory cytokines, the most likely candidates to mediate the circadian response to bacterial endotoxin. Moreover, it has been shown that there are specific receptors of other proinflammatory cytokines, such as interferon γ (IFNγ) in the SCN (Lundkvist et al., 1999). Indeed, high doses of an IFNγ–TNFα cocktail disrupt electrical activity of SCN neurons (Lundkvist et al., 2002).

As previously reported by Castel et al. (1993), light induced c-Fos expression through all the SCN (dorsal and ventral) while only the dorsal area was activated by LPS. This is in accordance with a suggested role for the dorsal SCN in synchronization by non-visual modulating inputs (Moga and Moore, 1997; Moore et al., 2002) and suggests that this region might be the anatomical substrate where immune-circadian interactions take place. This experiment also suggests that the SCN dorsal area may also function as an interaction area between light and endogenous entraining variables.

Almost all of the immune parameters exhibit diurnal or circadian rhythms either in their basal levels or after immune challenges. The physiological implications of these temporal variations remain unclear in most cases. One of the most studied physiological roles of immune variables in the central nervous system is the regulation of sleep by pro- and anti-inflammatory cytokines. It is now clear that proinflammatory cytokines induce sleep while anti-inflammatory cytokines prevent sleep induction (Krueger et al., 2001; Opp and Toth, 1998). LPS injections produce similar results to those of the proinflammatory cytokines on sleep regulation (Kapas et al., 1998) and exert differential effects on EEG activity in rats depending on the time of administration (Lancel et al., 1996). But, although the sleep–wake cycle is one of the clearest outputs of the circadian system, and its relationship with immune effectors has been clearly established, there are very few reports concerning the interactions between immune and circadian variables, most of which are in vitro approaches. The few in vivo experiments usually involve strong challenges to the immune system such as high doses of LPS or pathological states that severely affect circadian output (Bauhofer et al., 2002; Halberg et al., 1960). As for the in vitro results, Nava et al. (2000) reported the alteration of the circadian arginine-vasopressin release from SCN cultures after stimulation with LPS while Motzkus et al. (2002) have shown IL-6-induced *mper1* (a bona fide circadian clock genetic component) expression in cell cultures. The PVN response to immune challenges has been supported by a recent study which shows up regulation of the *mPer1* gene in the PVN CRF neurons by stress signals like LPS, forced swimming and immobilization (Takahashi et al., 2001).

Severe immune challenges such as animal models of sepsis (Bauhofer et al., 2001; Ebong et al., 1999), or infection with blood-borne parasites such as *Trypanosoma cruzi* or *T. brucei* (Bentivoglio et al., 1994), or even HIV-infected animals or patients (Bourin et al., 1993; Vagnucci and Winkelstein, 1993) have shown different levels of circadian disruption, including complete arrhythmicity, suggesting that circadian rhythms can be considered a good quality-of-life indicator (Mormont and Waterhouse, 2002). Indeed, the relationship between immune states and circadian rhythms might be useful in clinical situations where pro or anti-immune treatment is necessary. Interestingly, the administration of immunesuppressors like cyclosporin A or FK-506 induce non-photic like phase response curve of running wheel activity rhythms (Golombek et al., 1998) while an immune challenge produces photic-like phase delays as shown herein.

The results reported in the present work suggest a close interaction between the circadian and the immune systems. In this interaction, inflammation mediators such as proinflammatory cytokines and NF-κB transcription factors might be regulating the input and/or molecular machinery of the circadian clock. Indeed, it is tempting to speculate a mechanism through which clock-controlled humoral factors feedback upon the oscillator mechanism in order to fine-tune its timekeeping mechanisms. Still, much more evidence is needed to clearly understand which is the role of different cytokines within the circadian system and exactly how the administration of LPS can modify the molecular clock mechanism.

Acknowledgments

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