Effects of prior stress on LPS-induced cytokine and sickness responses

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Abstract

It has recently been reported that exposure to inescapable tailshock (IS) enhances the release of proinflammatory cytokines following bacterial challenge. However, it is not known whether the level of potentiation of proinflammatory cytokines is sufficient to exaggerate any of the physiological processes that are regulated by these cytokines. Thus, lipopolysaccharide (LPS) was administered and fever, activity, HPA responses, and proinflammatory cytokine release were assessed during both the light and dark phases of the light cycle following IS. Exposure to IS resulted in elevated basal core body temperature during the light phase but not the dark phase and decreased activity during the dark phase but not the light phase. IS animals had significantly greater fever, corticosterone, and ACTH responses following LPS during both the light and dark phases, while enhanced proinflammatory cytokine responses were only observed during the light phase. These data suggest that enhanced proinflammatory cytokine responses are not necessary to observe enhanced HPA or fever responses.
Introduction

Stress has long been implicated in the etiology of psychiatric disorders. More recently, alterations in immune system function have been suggested to play a role in the pathophysiology of psychiatric conditions such as major depression and anxiety (1, 12, 32, 49).

Recently, we reported cross-sensitization between stress and the production of proinflammatory cytokines (21, 22). In these studies, animals exposed to inescapable tailshock (IS) showed both enhanced peripheral and central induction of proinflammatory cytokines and HPA activation 24 h later when challenged with lipopolysaccharide (LPS; a component of the cell walls of gram negative bacteria). The release of proinflammatory cytokines such as interleukin-(IL-)1β, IL-6, and tumor necrosis factor-α (TNF-α) during infection are critical in initiating the inflammatory response needed for localization and elimination of invading pathogens (19).

In addition, proinflammatory cytokines signal the brain, leading to activation of regions involved in the neurally mediated components of host defense (4, 10). This aspect of host defense has been called the “sickness response” and includes fever, increased non-rapid eye movement (NREM) sleep, reductions in food and water intake, reduced exploration, reduced social behavior, hyperalgesia, HPA activation, and increased sympathetic nervous system activity (see (30) for review). It is the ability of cytokines to alter brain function (4, 10) and lead to depressive-like behaviors (5) that has implicated them in psychiatric diseases. The fact that experiencing stressful life events can exaggerate the release of proinflammatory cytokines to immune challenge suggests the possible importance of cross-sensitization in the etiology of affective disorders.
Besides sensitizing proinflammatory cytokine release in response to bacterial challenge, exposure to IS has been shown to increase cytokines for several hours (35), elevate positive acute phase proteins and decrease negative acute phase proteins (7), and alter the hypothalamic-pituitary-adrenal (HPA) axis as observed by elevated basal glucocorticoids (11) and glucocorticoid resistance (36) for several days following stressor exposure. Altered HPA function may be directly involved in the sensitization to immune challenge. Elevated basal levels of corticosterone (CORT) may enhance cytokine release as suggested by the finding that low levels of CORT enhance LPS-induced TNF release from perfused isolated rat liver (26) and glucocorticoid resistance may lead to enhanced proinflammatory cytokine release as suggested by the failure of dexamethasone, a synthetic glucocorticoid, to inhibit the release of cytokines following LPS administration in IS animals (36). These stress-induced changes are not specific to IS, as exposure to social reorganization and chronic tailshock also produce similar changes (2, 38, 48).

Interestingly, elevated proinflammatory cytokines (28), altered acute phase proteins (29), and dysregulation of the HPA axis (39) have all been reported in patients suffering from major depression.

The mechanism(s) and physiological significance of the stress-induced sensitization of cytokines are unknown. Thus, it is not known whether the level of potentiation of proinflammatory cytokines following immune challenge is sufficient to exaggerate any of the physiological processes that are regulated by these cytokines. To examine one aspect of possible physiological significance, LPS-induced fever was assessed following IS. Since proinflammatory cytokines are involved in the induction of fever, enhanced cytokine responses following stressor exposure may alter the fever
response and thus the organisms’ chance for survival. Fever was assessed following LPS administration during both the light and dark phases, since fever regulation and proinflammatory cytokine responses have been shown to differ between the day and night (40, 41, 45). Circadian differences in fever and proinflammatory cytokine responses may be due to shifts in circadian hormone secretions such as glucocorticoids and melatonin, both known to alter immune responses (34, 47). Exposure to IS has been reported to alter the circadian secretion of glucocorticoids (11), thus it is proposed that IS may differentially effect fever and proinflammatory cytokine release throughout the circadian cycle. Since enhanced proinflammatory cytokine release following immune challenge has only been characterized during the light phase, LPS-induced proinflammatory cytokine release was also assessed during both the light and dark phases.

It is known that IS will elevate core body temperature (CBT), but the details (duration and circadian rhythmicity) of this effect are unknown. Thus, in the present experiments we investigated the effects of IS on CBT in detail and whether exposure to IS alters the fever response to bacterial challenge. Fever was assessed by measurement of CBT by telemetry following either an injection of sterile, endotoxin-free saline or 10 µg/kg LPS (a dose previously found to result in a submaximal proinflammatory cytokine and HPA response (14)). Injections were made at either 0900 h (during the light phase) or 2200 h (during the dark phase) to determine whether there would be differential effects at different points of the circadian cycle and CBT and activity measured for 24 h. In addition, we investigated whether IS differentially alters LPS-induced cytokines during the light (0900 h) and dark phase (2200 h) and whether basal CORT values differ at these times. Thus, baseline blood samples were taken followed by LPS administration and
animals sacrificed 1 or 2 h later. Plasma IL-1β, TNF-α, IL-6, ACTH, and CORT were measured, along with IL-1β levels in various brain regions.
Materials and Methods

Subjects. Adult male Sprague Dawley rats (325-375 gms; Harlan Sprague Dawley, Inc., Indianapolis, IN) were individually housed in either plastic cages or hanging metal cages with food and water available *ad libidum*. Colony conditions were maintained at 25° C on a 12-h light, 12-h dark cycle (lights on at 08:00 h). Rats were given at least 2 wk to habituate to the colonies before experimentation. Care and use of animals were in accordance with protocols approved by the University of Colorado Institutional Animal Care and Use Committee.

Inescapable Tailshock (IS) protocol. Animals either remained in their home cages as controls (HCC) or were placed in Plexiglas tubes (23.4 cm length X 7 cm width) and exposed to 100 5-s, 1.6-mA inescapable tailshocks (IS), with an average intertrial interval of 60 s. All stress procedures occurred between 0800-1100 h. After stressor termination, rats were returned to their home cages.

Fever assessment. Rats were anesthetized with Isoflurane and emitters for measuring core body temperature (MiniMitter, Sun River, OR) were implanted in the peritoneal cavity as previously described (50). 4 wk recovery was allowed before testing. The fever response to LPS was assessed either during the light phase with injections occurring at 0900 h, or during the dark phase with injections occurring at 2200 h. Injections during the dark phase were made 2 h into the cycle to insure that the differences in baseline core body temperatures between IS and HCC animals were no longer present. Core body temperature was measured by telemetry every min and averaged over 15 min intervals. On Day 1 of the experiment, animals were injected with sterile, endotoxin-free saline (Abbott Laboratories, North Chicago, IL) and baseline core body temperatures recorded.
for 24 h. On Day 2, animals were either exposed to IS or served as HCCs. On Day 3, animals were injected with saline and core body temperatures recorded for 24 h. On Day 4, animals were injected with 10 μg/kg LPS (*Escherichia coli* endotoxin 0111:B4, Sigma lot#17H4041) and core body temperatures recorded for 24 h. On Day 5, animals were injected with saline and core body temperatures recorded for 24 h. Core body temperature recordings continued through Day 8, but since there were no differences in core body temperatures between HCC and IS animals on days 6, 7, and 8 post-IS the data were not presented. This design allowed for each animal to serve as its own control and a determination of the length of time exposure to IS shifts an animals’ basal core body temperature. Since approximately 4% of animals fail to have detectable levels of endotoxin or plasma cytokines following LPS administration, animals that failed to mount a fever response following LPS were eliminated from the study. Two of forty-five (4.4%) animals were dropped for failure to mount a fever response following LPS injection. Figure 1 outlines experimental design for fever studies.

**Activity.** Gross motor movement was assessed by telemetry using the same emitters used for recording core body temperature. The emitter had to move for activity to be counted, thus stationary movement such as grooming were not counted. Activity counts were measured every minute and averaged over 1 h intervals. Baseline activity data following IS are presented from animals involved in the light phase study only, while activity data following LPS are presented for both the light and dark phase studies.

**Plasma and tissue collection.** Blood samples were taken from animals either one day prior to and one day after IS at 0900 h and 2200 h for measurement of basal CORT or from animals immediately prior to LPS injection (0900 h or 2200 h) for measurement of
basal cytokines. To obtain baseline blood samples, the rat was removed from its home cage, gently wrapped in a towel, and lightly restrained with a Velcro strap. The tail was exposed and a small nick was made in a lateral tail vein with a scalpel (no. 15 blade), and the tail gently stroked until a volume of approximately 200-300 µl of whole blood was obtained in microfuge tubes. The entire sampling procedure was accomplished within 2 min of approaching the cage. Upon completion of blood collections, the samples were spun in a refrigerated centrifuge, and plasma was aliquoted and stored at −20°C until the time of assay. Animals were injected i.p. with 10 µg/kg LPS (*Escherichia coli* endotoxin 0111:B4, Sigma lot#17H4041) at either 0900 h or 2200 h, two days following exposure to IS, and sacrificed 1 or 2 h later. Trunk blood was collected in EDTA coated tubes for later measurement of adrenocorticotropin-releasing hormone (ACTH) and non-EDTA coated tubes for later measurement of cytokines, corticosterone, and endotoxin. Tubes were collected on ice and immediately spun in a refrigerated centrifuge upon completion of sampling. Plasma was aliquoted and stored at -80°C for later measurement of ACTH or stored at -20°C for later measurement of plasma cytokines and CORT. The pituitary and brain were quickly removed after decapitation. Brains were dissected on a frosted glass plate placed on top of crushed ice. Brain and pituitary samples were placed in microfuge tubes and quickly frozen in liquid nitrogen. These tissue samples were stored at −80°C until the time of sonication. Figure 2 outlines experimental design for studies assessing cytokine and CORT responses.

**Brain Tissue processing.** Each tissue was added to 0.25-1.0 ml of cold Iscove’s culture medium containing 5% fetal calf serum and a cocktail enzyme inhibitor (in mM: 100 amino-\(n\)-caproic acid, 10 EDTA, 5 benzamidine-HCl, and 0.2 phenylmethylsulfonyl
fluoride). Total protein was mechanically dissociated from tissue using an ultrasonic cell
disruptor (Heat Systems, Inc., Farmingdale, NY). Sonication consisted of 10 sec of cell
disruption at the setting 10. Sonicated samples were centrifuged at 14,000 rpm at 4°C for
10 min. Supernatants were removed and stored at 4°C until an ELISA was performed.
Bradford protein assays were also performed to determine total protein concentrations in
brain sonication samples.

Measurement of cytokines. Cytokines were measured using commercially available
ELISAs for rat IL-1β, TNF-α, and IL-6 (R & D Systems, Minneapolis, MN). The
ELISAs were run according to the manufacturer’s instructions. The rat IL-1β and TNF-α
kits have a detection limit of <5 pg/ml and the IL-6 kit has a detection limit of <8pg/ml.
The intra- and inter-assay variability is <10%.

Measurement of plasma endotoxin. Plasma levels of endotoxin were determined by an
enzymatic assay, according to the procedure outlined by Bio-Whittaker (cat# 50-648U;
Walkersville, MD). The detection limit of the assay is 0.02 EU/ml. Plasma was diluted
1:10. Animals that were injected with LPS, but had no detectable levels of plasma
endotoxin, also had no increase in plasma or brain cytokine levels compared to saline
injected controls. Presumably, injections were made into an internal organ, which
resulted in no detectable immune response. Therefore, these animals were eliminated
from the study. Approximately 4% of the animals were eliminated from the study due to
the absence of detectable endotoxin and were evenly distributed between groups.

Measurement of plasma corticosterone. Total plasma CORT levels were measured by
RIA. Plasma samples (20 µl) were diluted in 0.01 M PBS and heat inactivated for 1 h at
75°C. Samples and corticosterone standards (25-2000 pg/tube) were incubated overnight
with antiserum (rabbit antibody B21-42; Endocrine Sciences, Inc., Tarzana, CA) and [3-H] corticosterone (20,000 cpm/tube). Antibody-bound steroid was separated from free steroid with dextran-coated activated charcoal. The assay sensitivity was approximately 0.5 µg/ml for a 20 µl plasma sample. Interassay and intraassay coefficients of variation were less than 9%.

**Measurement of plasma ACTH.** Plasma levels of ACTH were determined by RIA. Plasma samples (50 µl) and ACTH standards (15.6 -1000 pg/ml) were incubated overnight at 4˚C with antiserum (rabbit antibody Rb7; courtesy of Dr. William Engeland, University of Minnesota) and 100 µl of [¹²⁵I] ACTH. 100 µl of goat anti-rabbit IgG (Calbiochem, La Jolla, CA, Cat # 539844) and 100 µl of normal rabbit serum (Vector Laboratories, Burlingame, CA, Cat # S-5000) was added and allowed to incubate for 30 min before adding 2 ml of 5% polyethylene glycol (Sigma). Tubes were spun for 30 min at 4000 rpm at 4°C, decanted and pelleted radioactivity was measured using a gamma counter. The assay sensitivity was approximately 10 pg/ml for a 50 µl plasma sample.

**Statistics.** The experiment examining basal corticosterone was analyzed using a 2 X 4 repeated measure ANOVA between stress condition (IS vs. HCC) and time of sampling (pre-IS AM vs. pre-IS PM vs. post-IS AM vs. post-IS PM). Experiments examining plasma cytokines, CORT, ACTH, and brain cytokines were analyzed using a 2 X 2 ANOVA between stress condition (IS vs. HCC) and Time of Day (0900 h vs. 2200 h). Post hoc analyses were done using a Fisher’s least significant difference test. Baseline core body temperature and activity data were analyzed using a repeated measure ANOVA between stress condition (IS vs. HCC) and time (twenty-four hours). Baseline core body temperatures differed between animals following IS exposure, but since each
animal served as its own control, a change from baseline was calculated to determine the effects of IS on fever induction. Recordings obtained on the post-IS control day (Day 3) were subtracted from the recording obtained at the same time on the test day (Day 4). A repeated measure ANOVA was done between stress condition and time using the change in body temperature following LPS, data were analyzed from the time of injection (0900 h or 2200 h) through the end of the light cycle, the approximate time the fever lasts. In all cases p<.05 was used for the level of confidence for acceptance of significance to exclude the null hypothesis.
Results

Effects of stress on the circadian rhythm of activity

Baseline activity for HCC and IS animals are shown in Fig. 3A-C. Animals showed normal circadian rhythm of activity with less activity during the light phase and more activity during the dark phase. Baseline activity prior to IS was identical between HCC and IS animals. Exposure to IS did not affect activity 1 day later during the light phase, but decreased activity during the dark phase. A repeated measure ANOVA revealed a significant interaction between stress and time of day \( F(23,598) = 2.055; p = .003 \). Post hoc analysis revealed that IS animals had significantly less activity during the dark phase \( F(1,26) = 23.37; p < .0001 \), but not during the light phase \( F(1,26) = .355; p = .556 \) 1 day post-IS. The decrease in activity of IS animals during the dark phase was no longer present 3 days post-IS \( (p = .144) \).

Effects of stress on the circadian rhythm of core body temperature

Basal core body temperature recordings for HCC and IS animals are shown in Fig. 3A-C. On all days animals showed normal circadian rhythm of body temperature with lower body temperatures during the light phase and higher body temperatures during the dark phase. Baseline core body temperatures prior to IS were identical between HCC and IS animals. Exposure to IS resulted in elevated basal core body temperature 1 day later during the light phase, but not the dark phase. A repeated measure ANOVA revealed a significant interaction between stress and time of day \( F(95,2470) = 2.947; p < .0001 \). Post hoc analysis revealed that IS animals had significantly elevated core body temperature during the light phase \( F(1,26) = 11.48; p = .002 \), but not the dark phase \( F(1,26) = .253; p = .619 \) 1 day post-IS. The shift in core body temperature during the
light phase persisted for 3 days post-IS [F(1,26) = 5.03; p = .034], but was no longer
significant on days 4, 5, or 6 post-IS (p = .153), (p = .621), and (p = .860), respectively
(Data not shown). Differences in body temperature during the dark phase were also
calculated using a MANCOVA to account for the difference in activity between IS and
HCC animals during this time. A repeated measures MANCOVA revealed a significant
difference in body temperature between stress conditions when analyzed using activity as
a covariate [F(1,332) = 7.36; p = .007].

Effects of Prior Stress on activity following LPS

Activity data following administration of LPS during the light and dark phases are
presented in Fig. 4A,B. The change in activity from baseline day 3 is presented in Fig.
4C,D. Injection of LPS during the light phase decreased activity of both IS and HCC
animals during the light phase and ensuing dark phase. Injection of LPS during the dark
phase only disrupted activity for a short amount of time with activity returning to normal
within the same phase. A repeated measure ANOVA revealed a significant change in
activity across time when LPS was injected during the light phase [F(11,286) = 7.55; p <
.0001]. A repeated measure ANOVA also revealed a significant change in activity
during the ensuing dark phase following injection of LPS [F(11,286) = 3.19; p = .0004].
There was also a significant main effect of stress (HCC vs. IS) during the ensuing dark
phase, with IS animals having significantly lower activity than HCCs [F(1,26) = 5.135; p
= .032]. When LPS was injected during the dark phase, again activity was suppressed
[F(11,143) = 4.77; p < .0001], but there was no difference between IS and HCC animals
(p = .129).
Effects of Prior Stress on LPS-induced fever

Administration of LPS increased CBT in a time dependent manner in all animals during both the light and dark phases (Fig. 5A,B). Animals previously exposed to IS had significantly higher CBTs in response to LPS regardless of the light cycle during which the LPS was administered. However, since IS animals had elevated basal core body temperatures during the light phase (see Fig. 3B) and our aim was to determine the effects of the sensitized cytokine response on fever, difference scores were analyzed using the body temperature recordings from the previous day (Day 3) (Fig. 5C,D). This diminished the difference in LPS induced fever observed during the light phase, but not the difference during the dark phase. A repeated measure ANOVA revealed no significant difference in the change in core body temperature between IS and HCC animals following LPS administration during the light phase [F(1,26) = 3.12; p = .089], but did reveal a significant enhancement during the ensuing dark phase [F(1,26) = 8.56; p = .007]. It should be noted that if the rising phase of the fever response (through the peak at timepoint 1400 h) were analyzed, which is the time at which enhanced cytokine release was previously observed in IS animals (22), IS significantly enhances the fever response [F(1,26) = 6.06; p = .021]. A repeated measure ANOVA revealed a significant difference in the change in core body temperature between IS and HCC animals following LPS administration during the dark phase (2200 h) [F(1,13) = 11.30; p = .005].

Effects of Stress on the diurnal levels of CORT

Basal CORT levels at 0900 h and 2200 h pre- & post-IS are shown in Fig. 6. All animals prior to IS showed a diurnal rhythm of CORT with low levels during the light phase and higher levels during the dark phase. Exposure to IS eliminated the diurnal
rhythm of CORT. IS animals had significantly elevated CORT levels during the light phase, but significantly lower CORT levels during the dark phase compared to HCC animals. A 2 X 4 repeated measure ANOVA revealed a significant interaction between stress (IS vs. HCC) and time (pre-IS AM vs. pre-IS PM vs. post-IS AM vs. post-IS PM) [F(3,102) = 7.025; p = .0002]. Post hoc analysis revealed no significant difference in CORT levels between stress condition prior to IS during the light phase (p = .669) or the dark phase (p = .446), but did reveal a significant difference between stress condition following IS during both the light phase (p = .003) and the dark phase (p = .027). Animals exposed to IS did not significantly differ in their CORT levels between the light and dark phase (p = .195).

*Effects of Stress on the diurnal levels of basal cytokines*

Basal circulating cytokine levels are shown in Fig. 7A-C. Basal levels of IL-1β and IL-6 were detectable in all groups and did not vary between phases of the light cycle. Basal TNF-α was undetectable in all groups at both times of the day. IS significantly decreased circulating basal levels of IL-1β during the light and dark phase, but had no effect on circulating IL-6. A 2 X 2 ANOVA revealed a significant effect of stress condition on basal IL-1β levels [F(1,25) = 16.22; p = .0005].

*Effects of Stress on LPS-induced circulating cytokines*

Circulating levels of proinflammatory cytokines following LPS are shown in Fig 7A-C. Plasma TNF-α levels peaked 1 h after LPS and were still elevated at 2 h post LPS. The phase of the light cycle in which LPS was injected did not affect TNF-α levels at 1 h, but significantly greater TNF-α levels were detected 2 h following LPS during the dark phase. Exposure to IS resulted in greater TNF-α levels during the light phase at
both 1 and 2 h post LPS, but had no effect on TNF-α levels during the dark phase. A 2 X 2 ANOVA revealed a significant effect of stress [F(1,32) = 8.149; p = .008] 1 h following LPS. Posthoc analysis revealed a significant effect of stress during the light phase (p=.0004), but not during the dark phase (p=.436). A 2 X 2 ANOVA revealed a significant effect of time of day [F(1,26) = 4.463; p = .044] 2 h following LPS. The effects of stress 2 h post LPS were not significance during the light (p=.059) or dark phase (p=.97). Plasma IL-1β was not elevated 1 h post LPS, but was significantly elevated at 2 h. Neither phase of the light cycle nor IS had any significant effect on circulating IL-1β levels. Plasma IL-6 levels were elevated 1 h post LPS and peaked at 2 h. Significantly greater levels of IL-6 were detected during the light phase 1 h following LPS, but not at 2 h. Exposure to IS resulted in significantly greater levels of plasma IL-6 at both 1 and 2 h following LPS during the light phase, but had no effect on IL-6 levels during the dark phase. A 2 X 2 ANOVA revealed a significant effects of time of day [F(1,31) = 13.903; p = .0008] and stress [F(1,31) = 4.523; p = .042] 1 h following LPS. Posthoc analysis revealed significantly lower levels of IL-6 1 h after LPS during the dark phase for both HCC (p=.041) and IS (p=.013) animals and significantly greater levels of IL-6 in IS animals during the light phase (p=.047) but not the dark phase (p=.597). A 2 X 2 ANOVA also revealed a significant effect of stress [F(1,26) = 4.964; p = .035] 2 h following LPS. Again, IS resulted in significantly greater IL-6 levels during the light phase (p=.009), but not the dark phase (p=.915).

**Effects of Stress on LPS-induced central IL-1β**

Central levels of IL-1β following LPS are shown in Fig 8A-D. Levels of IL-1β increased in all brain regions and pituitary from the 1 h to the 2 h timepoint, while the
effects of IS and phase of the light cycle on central IL-1β were specific to the individual brain area. Exposure to IS increased hypothalamic IL-1β at both 1 and 2 h following LPS during the light phase but not the dark phase. In control animals, hypothalamic IL-1β levels were significantly greater during the dark phase 2 h following LPS compared to the light phase, but these differences were not observed at 1 h or in the IS group. A 2 X 2 ANOVA reveal no significant effect of stress 1 h following LPS. A 2 X 2 ANOVA revealed a significant interaction between stress and time of day [F(1,25) = 7.359; p = .012] 2 h following LPS. Post hoc analysis revealed a significant difference between stress conditions during the light phase (p=.038) but not the dark phase (p=.117) and a significant difference between time of day for HCC animal (p=.040) but not for IS animals (p=.143). Exposure to IS had no effect on hippocampal IL-1β 1 h following LPS, but increased IL-1β levels 2 h post LPS. Phase of the light cycle in which LPS was administered had no effect on IL-1β levels. A 2 X 2 ANOVA revealed a significant effect of stress [F(1,25) = 5.50; p = .027] 2 h post LPS. Posthoc analysis revealed IS significantly increased hippocampal IL-1β during the light phase (p=.036) but not the dark phase (p=.722). Exposure to IS had no effect on cortical IL-1β at either timepoint following LPS, but IL-1β levels were significantly greater when LPS was administered during the light phase compared to the dark phase. A 2 X 2 ANOVA revealed a significant effect of time of day [F(1,31) = 12.29; p = .001] [F(1,25) = 9.707; p = .0046] at both the 1 and 2 h timepoints following LPS, respectively. Exposure to IS significantly increased pituitary IL-1β at 1 h, but was not significant at 2 h following LPS, while time of day in which the LPS was administered had no effect on IL-1β production. A 2 X 2 ANOVA revealed a significant effect of stress [F(1,30) = 4.437; p =
.044] 1 h following LPS. Posthoc analysis revealed a significant effect of stress during the light phase (p=.041) but not the dark phase (p=.74) 1 h post LPS and no significant effect of stress during the light phase (p=.125) or dark phase (p=.903) 2 h post LPS.

*Effects of Stress on LPS-induced CORT and ACTH*

Plasma CORT and ACTH levels following LPS are shown in Fig. 9A,B. In control animals, both CORT and ACTH increased from the 1 h to the 2 h timepoint following LPS. Exposure to IS significantly increased CORT and ACTH 1 and 2 h following LPS compared to controls and regardless of the light phase in which animals were injected. A 2 X 2 ANOVA revealed a significant effect of stress condition [F(1,32) = 58.131; p < .0001] [F(1,25) = 7.615; p = .011] on plasma CORT levels 1 and 2 h post LPS, respectively. Post hoc analysis revealed a significant effect of stress during both the light (p<.0001) and dark (p=.006) phase 1 h post LPS, but only during the light phase (p=.037) 2 h post LPS. A 2 X 2 ANOVA also revealed a significant effect of stress condition [F(1,31) = 45.965; p < .0001] [F(1,26) = 4.455; p = .045] on plasma ACTH levels 1 and 2 h post LPS, respectively. Post hoc analysis reveal a significant effect of stress during both the light (p<.0001) and dark (p=.009) phase 1 h post LPS, but neither were significant when analyzed separately 2 h post LPS.
Discussion

Normal circadian rhythm of body temperature and activity was observed in control animals with low levels measured during the light phase and higher levels measured during the dark phase of the light cycle. Exposure to IS disrupted normal circadian rhythm by reducing the amplitude of the difference in temperature and activity between the light and dark phase. For body temperature this was due to increased basal temperature during the light phase, but no subsequent increase during the dark phase and for activity it was due to decreased activity during the dark phase, but not during the light phase. These data support previous research showing increased basal temperature in IS animals during the light phase (7) and add the fact that temperatures do not differ during the dark phase but that activity does differ. The present data also add information concerning the timecourse of temperature change, finding that the effects of IS on CBT persist for 3 days. The same pattern of altered body temperature and activity has been observed following social defeat (31). In these experiments social defeat occurred during the dark phase suggesting that disturbing animals during the light cycle is not the cause of the altered circadian pattern.

It is possible that the IS induced decrease in activity during the dark phase is responsible for the finding that the basal shift in CBT was not observed during the dark phase. Increased activity during the dark phase has been shown to produce at least part of the diurnal increase in CBT (17, 42). Thus, decreased activity in IS animals compared to controls could have eliminated any shift in basal CBT during the dark phase. It is possible that if a basal shift in CBT were not actually present during the dark phase, that IS animals would then have had decreased CBTs compared to controls due to their
decreased activity. In fact, a significant difference in basal CBT during the dark phase was observed when activity was used as a covariate in the analysis.

Exposure to IS also disrupted the normal circadian rhythm of CORT. In controls CORT levels rose from the AM to the PM. However, in IS subjects, CORT was elevated in the AM and did not rise in the PM. Since CORT was only measured at one timepoint during each phase of the light cycle it is impossible to know if this truly represents a lack of circadian rhythm or just a disruption in the normal pattern. Again, these data support previous research showing increased basal CORT in IS animals during the light phase (11) and add the fact that CORT levels are lower than controls during the dark phase. Exposure to other stressors have shown similar circadian patterns of CORT (23, 37, 38). It might also be noted that the shift in basal AM CORT produced by IS persists for over 48 h, and so the absence of a PM difference cannot be ascribed to a dissipation of the effects of IS between the AM and PM assessments.

Exposure to IS significantly decreased basal levels of IL-1β while having no effect on basal levels of TNF-α or IL-6. Furthermore, IS enhanced both peripheral and central levels of cytokines following LPS administration during the light phase, but had no effect on cytokine levels during the dark phase. While cytokine sensitization has been previously reported during the light phase (22), the pattern of the sensitization observed here was slightly different. In this study, sensitization was not observed in circulating IL-1β or in the hippocampus and cortex 1 h following LPS as previously observed, yet sensitization was observed 2 h following LPS in various serum levels of cytokines and in many brain areas which had not been observed in previous studies. This difference may be due to the time between IS exposure and LPS administration. In previous studies we
examined sensitization 24 h following IS, while here it was examined 48 h following IS. The shift in time was done to determine the level of cytokines that would have occurred during the fever studies. Since the fever studies required baseline temperature recordings 24 h following IS, LPS had to be administered 48 h following IS.

Changes in sensitization phenomena across time are not unusual. For example, Schmidt et al. (1995) (44) examined arginine vasopressin (AVP) stores in neurons within the external zone of the median eminence following a single systemic administration of IL-1β. No change was observed until 11 days later when AVP increased and then slowly declined back to basal levels over the next two weeks. In addition, Hayley et al. (1999) (16) found that sensitization of sickness behaviors grows with increased time between initial exposure and reexposure to TNF-α, with maximum sensitization occurring at the longest interval tested, 28 days. Hayley has also found that different patterns of sensitization are observed with different measures (neurotransmitters, hormones, behavior). Some responses enhanced quickly following an initiating stimulus and other responses grew more slowly over time. Thus, differences in the sensitization pattern 48 h post-IS compared to 24 h post-IS are not surprising. However, the absence of sensitization during the dark phase of the light cycle is surprising and is a novel finding. Sensitization during the dark phase has not been previously assessed.

It is not likely that the AM-PM sensitization differences are attributable to sensitization having dissipated by the dark phase, since previous studies have observed the cytokine and HPA sensitization for at least 4 days post-IS (21, 22). In addition, it cannot be argued that the higher levels of CORT normally observed during the dark phase suppressed the sensitization since CORT levels did not differ in IS animals.
between the light and dark phase. More likely, the presence of cytokine sensitization depends on the circadian rhythm of other systems. Whether it is an increase in a substance during the light phase that facilitates sensitization or an increase during the dark phase that inhibits sensitization is unclear. Thus, further studies are needed to examine the mechanism by which IS sensitizes the cytokine response. However, it is clear that if elevated CORT and/or glucocorticoid resistance are involved in the sensitization of the cytokine response during the light phase, they are not sufficient to alter cytokines during the dark phase.

Sensitization of the HPA response following LPS was observed during both the light and dark phase in IS animals, suggesting that sensitization of the HPA response is not dependent on sensitization of the cytokine response as one might have supposed. Previous findings also suggested that the two sensitization phenomena are independent since exposure of IS animals to a subsequent non-immune stressor also resulted in an enhanced HPA response (21).

Exposure to IS resulted in a robust increase in the fever response following LPS that did not depend on the light cycle in which LPS was administered. CBTs increased to approximately 38.6°C for controls and 39.1°C for IS animals following LPS, irrespective of the light cycle in which LPS was administered. However, the amplitude of the increased CBT was greater during the light phase than the dark phase due to the difference in basal CBT at those times as commonly observed (27). During the light phase, when activity was equal between groups, the shift in basal CBT accounted for most of the increase observed in the fever response between IS and control animals. This, along with the finding that CBT reached the same maximum temperatures during
both phases of the light cycle suggests that the enhanced fever response occurs because IS increases the “set-point” of thermoregulatory neurons. This would explain why IS animals have increased basal temperatures during the light phase, equal basal temperatures during the dark phase despite decreased activity, and enhanced fevers during the dark phase even though systemic and brain cytokines were equal between groups.

Even after compensation for the shift in basal body temperature during the light phase, IS animals still showed an enhanced early phase fever response compared to controls. The enhanced early phase of the fever response may be due to the enhanced production of proinflammatory cytokines at this time. It has been shown that the dose of LPS determines the length of the fever response, not the magnitude of the response, even though circulating and brain levels of cytokines increase in a dose dependent fashion (13). Thus, it would not be surprising if the IS-induced enhanced cytokine response facilitated early fever responses without altering the peak response.

The observation of sensitized proinflammatory cytokines and fever responses in the presence of elevated basal and enhanced stimulated levels of glucocorticoids is of particular interest, since glucocorticoids are known to suppress proinflammatory cytokines and reduce fever responses to LPS challenge [Morrow, 1993 #382; McClellan, 1994 #383; (33). The fact that the enhanced CORT response did not suppress the cytokine or fever response in IS animals may be due to resistance to the suppressive effects of glucocorticoids in IS animals (36).

The changes induced by IS reported here may be beneficial. The overall increase in the fever response in IS animals could be potentially adaptive since increased core
Body temperatures have been shown to result in a more rapid neutrophil migration and secretion of antibacterial chemicals, enhanced actions of interferons, and increased survival rates of lizards, goldfish and newborn mammals (see Kluger, 1986 (24) for review). While high fevers (greater than 40.5°C) can have detrimental effects on the host, including dehydration, delirium, focal lesions of certain organs, cardiopulmonary strain, and negative nutrient balance (3), the maximum core body temperature reached here was well below the temperatures required for these effects.

It is unclear whether the sensitized cytokine and fever responses have a functional role. A sensitized proinflammatory cytokine response following a stressor could be beneficial in localizing and eliminating possible bacterial infections as suggested by the finding that IS animals show facilitated recovery from subcutaneous bacterial challenge (8). Conversely, sensitized cytokine release could result in or aggravate problems that are known to be immune related, such as meningitis (18), respiratory distress (20), arthritis (43) and septic shock (15). Therefore, the enhanced cytokine response observed in IS animals may be beneficial in protecting an animal from low-grade infectious challenge, but deleterious in increasing the likelihood of other clinical manifestations.

Finally, it might be noted that changes in cytokine responses, like many of those reported following IS, have been observed in depressed patients (9, 25, 28, 29, 39, 46). Altered cytokine responses has been considered to be involved in the pathophysiology of depression since elevated levels of proinflammatory cytokines can lead to depressed mood (5) and anxiety (6). The data presented here suggest that if stress-induced alterations in cytokine responses occur in depressed patients, possibly involved in the
etiology of depression, then time of day in which cytokine parameters are assessed may be critical.
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References


Figure Captions

Figure 1: Schematic diagram of the experimental design for experiments assessing the effects of inescapable tailshock (IS) exposure on the circadian rhythm and LPS-induced changes in activity and core body temperature. Changes in activity and core body temperature were assessed following LPS administration during both the light and dark phase of the light cycle. All saline and LPS injections occurred at 0900 h for assessment of activity and core body temperature during the light phase (A) and at 2200 h for assessment during the dark phase (B). No injections occurred after Day 5.

Figure 2: Schematic diagram of the experimental design for experiments assessing the effects of inescapable tailshock (IS) exposure on the circadian rhythm of corticosterone (CORT) and LPS-induced proinflammatory cytokines, CORT, and adrenocorticotropin hormone (ACTH). In one study, blood samples were taken to assess the effects of IS on the circadian rhythm of CORT and proinflammatory cytokine responses 1 h following LPS administration (A). In a second study, blood samples were taken to assess the effects of IS on basal plasma proinflammatory cytokine levels and proinflammatory cytokine responses 2 h following LPS administration (B).

Figure 3: Circadian rhythm of activity and core body temperature in animals either exposed to inescapable tailshock (IS) or served as home cage controls (HCC). Activity and temperatures measurements were recorded for 24 h following injection of sterile saline at 0900 h 1 Day pre-IS (A); 1 Day post-IS (B); and 3 Days post-IS (C). Data points represent means +/- standard errors. Shaded region represents lights-out.

Figure 4: Activity in inescapable tailshock (IS) and home cage control (HCC) animals following administration of LPS either during the light phase (0900 h) (A,C) or during
the dark phase (2200 h) (B,D). Graphs A & B represent raw activity count data, while
graphs C & D represent the change in activity from baseline Day 3 (1 Day post-IS). Data
points represent means +/- standard errors. Shaded region represents lights-out.

**Figure 5:** Core body temperature in inescapable tailshock (IS) and home cage control
(HCC) animals following administration of LPS either during the light phase (0900 h)
(A,C) or during the dark phase (2200 h) (B,D). Graphs A & B represent raw core body
temperature data, while graphs C & D represent the change in body temperature from
baseline Day 3 (1 Day post-IS). Data points represent means +/- standard errors. Shaded
region represents lights-out.

**Figure 6:** Circulating plasma corticosterone (CORT) in inescapable tailshock (IS) and
home cage control (HCC) animals at 0900 h and 2200 h 1 Day pre-IS and 1 Day post-IS.
Data points represent means +/- standard errors. *, represents a significant difference
from AM levels (p < .0001). #, represents a significant difference from HCC levels
during the same time of day (p < .05).

**Figure 7:** Plasma levels of TNF-α (A), IL-1β (B), and IL-6 (C) in inescapable tailshock
(IS) and home cage control (HCC) animals 0, 1, and 2 h following i.p. administration of
LPS at either 0900 h (AM) or 2200 h (PM). Data points represent means +/- standard
errors. ND, represents non-detectable values. *, represents a significant difference from
HCC levels during the same time of day (p < .05).

**Figure 8:** IL-1β levels in the hypothalamus (A), hippocampus (B), cortex (C), and
pituitary (D) in inescapable tailshock (IS) and home cage control (HCC) animals 1 and 2
h following i.p. administration of LPS at either 0900 h (AM) or 2200 h (PM). Data
points represent means +/- standard errors. *, represents a significant difference from HCC levels during the same time of day (p < .05).

**Figure 9:** Circulating plasma CORT (A) and adrenocorticotropin (ACTH) (B) in inescapable tailshock (IS) and home cage control (HCC) animals 1 and 2 h following i.p. administration of LPS at either 0900 h (AM) or 2200 h (PM). Data points represent means +/- standard errors. *, represents a significant difference from HCC levels during the same time of day (p < .05).
Fig. 1

### A. Day 0
0900 h, Saline injection

### B. Day 0
2200 h, Saline injection

| Day 1 | 0900 h | No injection | Day 2 | 0900 h | IS (n=13) HCC (n=15) | Day 3 | 0900 h | saline injection | Day 4 | 0900 h | 10 µg/kg LPS | Day 5 | 0900 h | No injection | Day 6 | 0900 h | No injection | Day 7 | 0900 h | No injection | Day 8 | 0900 h | No injection |
|-------|--------|--------------|-------|--------|----------------------|-------|--------|-----------------|-------|--------|--------------|-------|--------|--------------|-------|--------|--------------|-------|--------|--------------|-------|--------|--------------|-------|--------|--------------|

Day 1 (0900 h) Saline injection

Day 2 (0900 h) IS (n=13) HCC (n=15)

Day 3 (0900 h) Saline injection

Day 4 (0900 h) 10 µg/kg LPS

Day 5 (0900 h) No injection

Day 6 (0900 h) No injection

Day 7 (0900 h) No injection

Day 8 (0900 h) No injection
Fig. 2

A. Day 1  Day 2  Day 3  Day 4
Blood Sample IS (n=17)  Blood Sample 10 μg/kg LPS 1 h Sacrifice
(0900 h & 2200 h)  HCC (n=19) (0900 h & 2200 h)

B. Day 1  Day 2  Day 3  Day 4
IS (n=15) Blood Sample 10 μg/kg LPS 2 h Sacrifice
HCC (n=15) (0900 h or 2200 h) (0900 h or 2200 h)
Fig. 4

A. Activity Counts following 10 μg/kg LPS

B. Activity Counts following 10 μg/kg LPS

C. Change in Activity Counts from 1 Day Post-IS

D. Change in Activity Counts from 1 Day Post-IS
Fig. 5

A. Raw Core Body Temperature following 10 μg/kg LPS

B. Raw Core Body Temperature following 10 μg/kg LPS

C. Change in Core Body Temperature from 1 Day Post-IS

D. Change in Core Body Temperature from 1 Day Post-IS
Fig. 6

**Plasma CORT Pre-IS**

- **HCC**
- **IS**

**Plasma CORT Post-IS**

- **HCC**
- **IS**
Fig. 7

A. Plasma TNF-α

B. Plasma IL-1β

C. Plasma IL-6

- **HCC**
- **IS**

Notes:
- ND: Not detected
- *: Significant difference from control
- **: Highly significant difference from control
Fig. 8

A. Hypothalamus

B. Hippocampus

C. Cortex

D. Pituitary
Fig. 9

A. Plasma CORT

B. Plasma ACTH