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

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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 hypothalamic-pituitary-adrenal (HPA) activation 24 h later when challenged with LPS (a component of the cell walls of gram-negative bacteria). The release of proinflammatory cytokines such as IL-1β, IL-6, and TNF-α during infection is 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 sleep, reductions in food and water intake, reduced exploration, reduced social behavior, hyperalgesia, HPA activation, and increased sympathetic nervous system activity (see Ref. 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 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

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FEVER SENSITIZATION

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. Because 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 affect fever and proinflammatory cytokine release throughout the circadian cycle. Because 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 (during the light phase) or 2200 (during the dark phase) to determine whether there would be differential effects at different points of the circadian cycle on CBT and activity measured for 24 h. In addition, we investigated whether IS differentially alters LPS-induced cytokines during the light (0900) and dark (2200) phases and whether basal Cort values differ at those times. Thus, baseline blood samples were taken followed by LPS administration and animals were killed 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 g; Harlan Sprague Dawley, Indianapolis, IN) were individually housed in either plastic cages or hanging metal cages with food and water available ad libitum. Colony conditions were maintained at 25°C on a 12:12-h light-dark cycle (lights on at 0800). 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.

IS protocol. Animals either remained in their home cages as controls (HCC) or were placed in Plexiglas tubes (23.4-cm length, 7.7-cm width) and exposed to 100 5-s, 1.6-mA IS, with an average intertrial interval of 60 s. All stress procedures occurred between 0800 and 1100. After stressor termination, rats were returned to their home cages.

Fever assessment. Rats were anesthetized with Isoflurane, and emitters for measuring CBT (MiniMitter, Sun River, OR) were implanted in the peritoneal cavity as previously described (50). Four-week recovery was allowed before testing. The fever response to LPS was assessed either during the light phase with injections occurring at 0900 or during the dark phase with injections occurring at 2200. Injections during the dark phase were made 2 h into the cycle to ensure that the differences in baseline CBTs between IS and HCC animals were no longer present. CBT was measured by telemetry every minute 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 CBTs were 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 CBTs were recorded for 24 h. On day 4, animals were injected with 10 μg/kg LPS (Escherichia coli endotoxin 0111:B4, Sigma lot#17H4041) and CBTs were recorded for 24 h. On day 5, animals were injected with saline and CBTs were recorded for 24 h. CBT recordings continued through day 8, but since there were no differences in CBTs between IS and HCC 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 animal’s basal CBT. Because ~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 45 (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 CBT. The emitter had to move for activity to be counted; thus stationary movements 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, whereas 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 1 day before and 1 day after IS at 0900 and 2200 for measurement of basal Cort or from animals immediately before LPS injection (0900 or 2200) 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 was gently stroked until a volume of ~200–300 µl of whole blood was obtained in microfuge tubes. The entire sampling procedure was accomplished within 2 min of approaching the cage. On 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 intraperitoneally with 10 µg/kg LPS (E. coli endotoxin 0111:B4, Sigma #H1204-4) at either 0900 or 2200, 2 days following exposure to IS, and killed 1 or 2 h later. Trunk blood was collected in EDTA-coated tubes for measurement of ACTH and non-EDTA-coated tubes for later measurement of cytokines, Cort, and endotoxin. Tubes were collected on ice and immediately spun in a refrigerated centrifuge on 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, Farmingdale, NY). Sonication consisted of 10 s of cell disruption at 4°C. Supernatants were removed and stored at 4°C until the time of sonication. 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 <8 pg/ml. The intra- and interassay variabilities are <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 with 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 Cort.** Total plasma Cort levels were measured by radioimmunoassay following LPS. Data were acquired in 0.01 M PBS and heat inactivated for 1 h at 75°C. Samples and Cort standards (25–2,000 pg/tube) were incubated overnight with antiserum (rabbit antibody B21–42; Endocrine Sciences, Tarzana, CA) and [3H]Cort (20,000 cpm/tube). Antibody-bound steroid was separated from free steroid with dextran-coated activated charcoal. The assay sensitivity was ~0.5 µg/ml for a 20-µl plasma sample. Interassay and intra-assay coefficients of variation were ~9%.

**Measurement of plasma ACTH.** Plasma levels of ACTH were determined by RIA. Plasma samples (50 µl) and ACTH standards (15.6–1,000 pg/ml) were incubated overnight at 4°C with antiserum (rabbit antibody Rb7; courtesy of Dr. W. Engeland, University of Minnesota) and 100 µl of [125I]-ACTH. One-hundred microliters of goat anti-rabbit IgG (Calbiochem, La Jolla, CA, Cat #53984-4) and 100 µl of normal rabbit serum (Vector Laboratories, Burlingame, CA, Cat #S-5000) were added and allowed to incubate for 30 min before adding 2 ml of 5% polyethylene glycol (Sigma). Tubes were spun for 30 min at 4,000 rpm at 4°C and decanted, and pellet radioactivity was measured using a gamma counter. The assay sensitivity was ~10 pg/ml for a 50-µl plasma sample.

**Statistics.** The experiment examining basal Cort was analyzed using a 2 × 4 repeated-measure ANOVA between stress conditions (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 × 2 ANOVA between stress conditions (IS vs. HCC) and time of day (0900 vs. 2200). Post hoc analyses were done using a Fisher’s least significant difference test. Baseline CBT and activity data were analyzed using a repeated-measure ANOVA between stress conditions (IS vs. HCC) and time (24 h). Baseline CBTs differed between animals following IS exposure, but because 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 conditions and time using the changes in body temperature following LPS. Data were analyzed from the time of injection (0900 or 2200) through the end of the light cycle and the approximate time the fever lasts. In all cases, P < 0.05 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 is shown in Fig. 3, A–C. Animals showed normal circadian rhythm of activity with less activity during the light phase and more activity during the dark phase. Baseline activity before 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 \([F(23,598) = 2.055; P = 0.003]\). Post hoc analysis revealed that IS animals had significantly less activity during the dark phase \([F(1,26) = 23.37; P = 0.0001]\) but not during the light phase \([F(1,26) = 0.355; P = 0.556]\) 1 day post-IS. The decrease in activity of IS animals during the dark phase was no longer present 3 days post-IS \((P = 0.144)\).

Effects of stress on the circadian rhythm of CBT. Basal CBT recordings for HCC and IS animals are shown in Fig. 3, A–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 CBTs before IS were identical between HCC and IS animals. Exposure to IS resulted in elevated basal CBT 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 \([F(95,2,470) = 2.947; P < 0.0001]\). Post hoc analysis revealed that IS animals had significantly elevated CBT during the light phase \([F(1,26) = 11.48; P = 0.002]\) but not the dark phase \([F(1,26) = 0.253; P = 0.619]\) 1 day post-IS. The shift in CBT during the light phase persisted for 3 days post-IS \([F(1,26) = 5.03; P = 0.034]\) but was no longer significant on days 4, 5, or 6 post-IS \((P = 0.153; P = 0.621; \text{and } P = 0.860)\), respectively (data not shown). Differences in body temperature during the dark phase were also calculated using a multivariate analysis of covariance (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 \([F(1,332) = 7.36; P = 0.007]\).

Effects of prior stress on activity following LPS. Activity data following administration of LPS during the light and dark phases are presented in Fig. 4, A and B. The change in activity from baseline day 3 is presented.
in Fig. 4, C and 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 \[ F(11,286) = 7.55; P < 0.0001 \]. A repeated-measure ANOVA also revealed a significant change in activity during the ensuing dark phase following injection of LPS \[ F(11,286) = 3.19; P = 0.0004 \]. There was also a significant main effect of stress (HCC vs. IS) during the ensuing dark phase, with IS animals having significantly higher activity than HCCs \[ F(1,26) = 5.135; P = 0.032 \]. When LPS was injected during the dark phase, again activity was suppressed \[ F(11,143) = 4.77; P < 0.0001 \], but there was no difference between IS and HCC animals \( P = 0.129 \).

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. 5, A and 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, because IS animals had elevated basal CBTs during the light phase (see Fig. 3B) and our aim was to determine the effects of the sensi-

Fig. 4. Activity in IS and HCC animals following 10-μg/kg administration of LPS either during the light phase (0900) (A, C) or during the dark phase (2200) (B, D). A and B: raw activity count data. C and D: change in activity from baseline day 3 (1 day post-IS). Data points represent means ± SE. Shaded region represents lights-out.

Fig. 5. Core body temperature in IS and HCC animals following 10-μg/kg administration of LPS either during the light phase (0900) (A, C) or during the dark phase (2200) (B, D). A and B: raw core body temperature data. C and D: change in body temperature from baseline day 3 (1 day post-IS). Data points represent means ± SE. Shaded region represents lights-out.
tized cytokine response on fever, difference scores were analyzed using the body temperature recordings from the previous day (day 3) (Fig. 5, C and 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 CBT between IS and HCC animals following LPS administration during the light phase \( F(1,26) = 3.12; P = 0.089 \) but did reveal a significant enhancement during the ensuing dark phase \( F(1,26) = 8.56; P = 0.007 \). It should be noted that if the rising phase of the fever response (through the peak at time point 1400) were analyzed, which is the time at which enhanced cytokine release was previously observed in IS animals (22), IS significantly enhances the fever response \( F(1,26) = 6.06; P = 0.021 \). A repeated-measure ANOVA revealed a significant difference in the change in CBT between IS and HCC animals following LPS administration during the dark phase \( 2200 \) \( F(1,13) = 11.30; P = 0.005 \).

**Effects of stress on the diurnal levels of Cort.** Basal Cort levels at 0900 and 2200 pre- and post-IS are shown in Fig. 6. All animals before 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 with HCC animals. A 2 \( \times \) 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) \( F(3,102) = 7.025; P = 0.0002 \). Post hoc analysis revealed no significant difference in Cort levels between stress conditions before IS during the light phase \( P = 0.669 \) or the dark phase \( P = 0.446 \) but did reveal a significant difference between stress conditions following IS during both the light phase \( P = 0.003 \) and the dark phase \( P = 0.027 \). Animals exposed to IS did not significantly differ in their Cort levels between the light and dark phases \( P = 0.195 \).

**Effects of stress on the diurnal levels of basal cytokines.** Basal circulating cytokine levels are shown in Fig. 7, A-C. Basal levels of IL-1\( \beta \) and IL-6 were detectable in all groups and did not vary between phases of the light cycle. Basal TNF-\( \alpha \) was undetectable in all groups at both times of the day. IS significantly decreased circulating basal levels of IL-1\( \beta \) during the light and dark phases but had no effect on circulating IL-6. A 2 \( \times \) 2 ANOVA revealed a significant effect of stress condition on basal IL-1\( \beta \) levels \( F(1,25) = 16.22; P = 0.0005 \).
Effects of stress on LPS-induced circulating cytokines. Circulating levels of proinflammatory cytokines following LPS are shown in Fig. 7, A-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 × 2 ANOVA revealed a significant effect of stress \( F(1,32) = 8.149; P = 0.008 \) 1 h following LPS. Post hoc analysis revealed a significant effect of stress during the light phase \( (P = 0.0004) \) but not during the dark phase \( (P = 0.436) \). A 2 × 2 ANOVA revealed a significant effect of time of day \( F(1,26) = 4.463; P = 0.044 \) 2 h following LPS. The effects of stress 2 h post-LPS were not significant during the light \( (P = 0.059) \) or dark phase \( (P = 0.97) \). 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 × 2 ANOVA revealed a significant effect of time of day \( F(1,31) = 13.903; P = 0.0008 \) and stress \( F(1,31) = 4.523; P = 0.042 \) 1 h following LPS. Post hoc analysis revealed significantly lower levels of IL-6 1 h after LPS during the dark phase for both HCC \( (P = 0.041) \) and IS \( (P = 0.013) \) animals and significantly greater levels of IL-6 in IS animals during the light phase \( (P = 0.047) \) but not the dark phase \( (P = 0.597) \). A 2 × 2 ANOVA also revealed a significant effect of stress \( F(1,26) = 4.964; P = 0.035 \) 2 h following LPS. Again, IS resulted in significantly greater IL-6 levels during the light phase \( (P = 0.009) \) but not the dark phase \( (P = 0.915) \).

Effects of stress on LPS-induced central IL-1β. Central levels of IL-1β following LPS are shown in Fig. 8, A-D. Levels of IL-1β increased in all brain regions and pituitary from the 1- to the 2-h time point, 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 with the light phase, but these differences were not observed at 1 h or in the IS group. A 2 × 2 ANOVA revealed no significant effect of stress 1 h following LPS. A 2 × 2 ANOVA revealed a significant interaction between stress and time of day \( F(1,25) = 7.359; P = 0.012 \) 2 h following LPS. Post hoc analysis revealed a significant difference between stress conditions during the light phase \( (P = 0.038) \) but not the dark phase \( (P = 0.117) \) and a significant difference between time of day for HCC animal \( (P = 0.040) \) but not for IS animals \( (P = 0.143) \). 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 × 2 ANOVA revealed a significant effect of stress \( F(1,25) = 5.50; P = 0.027 \) 2 h post-LPS. Post hoc analysis revealed IS signifi-

Fig. 8. IL-1β levels in the hypothalamus (A), hippocampus (B), cortex (C), and pituitary (D) in IS and HCC animals 1 and 2 h following intraperitoneal administration of LPS at either 0900 (AM) or 2200 (PM). Data points represent means ± SE. *Significant difference from HCC levels during the same time of day \( (P < 0.05) \).

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cantly increased hippocampal IL-1β during the light phase \((P = 0.036)\) but not the dark phase \((P = 0.722)\). Exposure to IS had no effect on cortical IL-1β at either time point following LPS, but IL-1β levels were significantly greater when LPS was administered during the light phase compared with the dark phase. A 2 × 2 ANOVA revealed a significant effect of time of day \([F(1,31) = 12.29; P = 0.001]\) \([F(1,25) = 9.707; P = 0.0046]\) at both the 1- and 2-h time points following LPS, respectively. Exposure to IS significantly increased pituitary IL-1β at 1 h but was not significant at 2 h following LPS, whereas time of day in which the LPS was administered had no effect on IL-1β production. A 2 × 2 ANOVA revealed a significant effect of stress \([F(1,30) = 4.437; P = 0.044]\) 1 h following LPS. Post hoc analysis revealed a significant effect of stress during the light phase \((P = 0.041)\) but not the dark phase \((P = 0.74)\) 1 h post-LPS and no significant effect of stress during the light phase \((P = 0.125)\) or dark phase \((P = 0.903)\) 2 h post-LPS.

Effects of stress on LPS-induced Cort and ACTH. Plasma Cort and ACTH levels following LPS are shown in Fig. 9, A and B. In control animals, both Cort and ACTH increased from the 1- to the 2-h time point following LPS. Exposure to IS significantly increased Cort and ACTH 1 and 2 h following LPS compared with controls regardless of the light phase in which animals were injected. A 2 × 2 ANOVA revealed a significant effect of stress condition \([F(1,32) = 58.131; P < 0.0001]\) \([F(1,25) = 7.615; P = 0.011]\) on plasma Cort levels 1 and 2 h post-LPS, respectively. Post hoc analysis revealed a significant effect of stress during both the light \((P < 0.0001)\) and dark \((P = 0.006)\) phases 1 h post-LPS but only during the light phase \((P = 0.037)\) 2 h post-LPS. A 2 × 2 ANOVA also revealed a significant effect of stress condition \([F(1,31) = 45.965; P < 0.0001]\) \([F(1,26) = 4.455; P = 0.045]\) on plasma ACTH levels 1 and 2 h post-LPS, respectively. Post hoc analysis revealed a significant effect of stress during both the light \((P < 0.0001)\) and dark \((P = 0.009)\) phases 1 h post-LPS, but neither was 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 phases. 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 time course 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 with 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, then IS animals would have had decreased CBTs compared with 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 AM to PM. However, in IS subjects, Cort was elevated in AM and did not rise in PM. Because Cort was only measured at one time point 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 has 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 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. Although 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 that 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. Because 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. (44) examined 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 2 wk. In addition, Hayley et al. (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 with 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, because 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 phases. 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 phases 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 nonimmune 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 ~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 with 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 (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 CBTs 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 (24). Although 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 CBT 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 have 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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