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PRIOR LAPAROTOMY OR CORTICOSTERONE POTENTIATES LIPOPOLYSACCHARIDE-INDUCED FEVER AND SICKNESS BEHAVIORS

Leah E. Hains[A,B,*], **Lisa C. Loram**[B,*], **Frederick R Taylor**[B], **Keith A Strand**[B], **Julie L. Wieseler**[B], **Ruth M. Barrientos**[B], **Jennifer J. Young**[B,C], **Matthew G Frank**[B], **Julia Sobesky**[B], **Thomas J. Martin**[D,E], **James C. Eisenach**[D,E], **Steven F. Maier**[B], **John D. Johnson**[F], **Monika Fleshner**[G], and **Linda R. Watkins**[B]

[A]Wadsworth Center, Albany, NY 12201-0509, USA

[B]Department of Psychology and the Center for Neuroscience, University of Colorado at Boulder, Boulder CO 80309-0345, USA

[C]Weill Cornell/Rockefeller/Sloan-Kettering Tri-Institutional MD-PhD Program, New York, New York, 10021, USA

[D]Department of Physiology and Pharmacology, Wake Forest University School of Medicine, Winston-Salem, NC 27175, USA

[E]Department of Anesthesiology Wake Forest University School of Medicine, Winston-Salem, NC 27175, USA

[F]Biological Sciences Department, Kent State University, Kent, OH 44242, USA

[G]Department of Integrative Physiology and the Center for Neuroscience, University of Colorado at Boulder, Boulder CO 80309-0345, USA

Abstract

Stimulating sensitized immune cells with a subsequent immune challenge results in potentiated pro-inflammatory responses translating into exacerbated sickness responses (i.e. fever, pain and lethargy). Both corticosterone (CORT) and laparotomy cause sensitization, leading to enhanced sickness-induced neuroinflammation or pain (respectively). However, it is unknown whether this sensitization affects all sickness behaviors and immune cell responses equally. We show that prior CORT and prior laparotomy potentiated LPS-induced fever but not lethargy. Prior CORT, like prior laparotomy, was able to potentiate sickness-induced pain. Release of nitric oxide (NO) from peritoneal macrophages stimulated *ex vivo* demonstrates that laparotomy, but not CORT sensitizes these cells.

To whom correspondence should be addressed: Lisa C. Loram, Muenzinger Psychology Building, Rm D-244, Dept of Psychology, Campus Box 345, University of Colorado at Boulder, Boulder, CO 80309-0345, Ph: 303-492-7034, Fax: 303-492-2967, lisa.loram@colorado.edu.
*Authors contributed equally

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1. INTRODUCTION

Communication of pathogen recognition from the peripheral immune system to the central nervous system (CNS) creates sickness responses, including fever, increased sleep, decreased food and water intake, social avoidance, decreased sexual drive and enhanced pain (Watkins and Maier, 2000). Extensive study of sickness responses has revealed that peripheral production of pro-inflammatory cytokines, especially interleukin-1β (IL-1β), by activated monocytes and macrophages is a key component in the induction of immune-to-CNS communication that generates sickness responses, including fever, lethargy and enhanced pain (Luheshi et al., 1993; Maier et al., 1993). Interestingly, immune-to-brain communication is not invariant, but rather can be influenced by the organism's prior history (Dantzer, 2001). One example of this is "priming", whereby an initial CNS or peripheral immune challenge induces a sensitized state such that responses to later challenges are amplified. Initial challenges, including injection of an adjuvant or lipopolysaccharide (LPS), acute stress, morphine withdrawal, prion infection, and advancing age, can enhance responses to later challenge with peripheral LPS, including enhanced production of peripheral and/or central pro-inflammatory cytokines and exaggerated sickness behaviors (Cahill et al., 1998; Combrinck et al., 2002; Feng et al., 2005; Johnson et al., 2002; Parant et al., 1995).

We have recently shown that two such priming events, a prior surgery that mimics an exploratory laparotomy and glucocorticoids (corticosterone [CORT] in rats) that mimics an acute stressor, result in potentiated glial responsivity within the CNS after subsequent LPS (Frank et al., 2010; Hains et al., 2010). Laparotomy, a rat model of abdominal exploratory surgery with manipulation of the abdominal viscera and musculature, causes persistent expression of the microglial activation marker CD11b in spinal cord dorsal horn (Hains et al., 2010). Even two weeks after laparotomy, when the allodynia is resolved, the sickness-induced pain response to intraperitoneal (i.p.) injection of lipopolysaccharide, (LPS) is potentiated (Hains et al., 2010). Further, our findings indicate that expression of this primed pain response depends on the persistent activation of spinal microglia initiated by laparotomy (Hains et al., 2010).

The second such challenge is prior administration of glucocorticoids. While glucocorticoids are classically known to be anti-inflammatory, recent evidence from our laboratory and others (Alexander et al., 2009; Frank et al., 2010; Loram et al., 2011; Sorrells et al., 2009) have identified that when CORT is administered before a subsequent immune challenge, the proinflammatory response is potentiated. CORT administered 24 h before but not coadministered or given after an LPS challenge resulted in potentiation of the microglial proinflammatory response and elevated CD11b gene expression in the hippocampus (Frank et al., 2010). Therefore, challenge with both laparotomy and CORT have been identified to sensitize central immune cells or glia. Whether this sensitization translates to modified sickness behaviors remains to be elucidated.

While we know that prior laparotomy is able to potentiate LPS-induced pain responses, we do not know if CORT is able to induce the same change in sickness-induced pain. It is also unknown whether sensitization by these two challenges is unique to sickness-induced pain responses or whether other aspects of the sickness response such as fever and lethargy would also be affected.

Lastly, both laparotomy and CORT (Haugen and Wiik, 1997) have been reported to cause acute immune suppression (Moehrlen et al., 2005; Pitombo et al., 2006), with surgery causing an increase in serum CORT measured 2 d later (Ogasawara et al., 1999). However it is unknown how peripheral immune responses to systemic LPS may be affected weeks after laparotomy or 24 h after CORT. We considered the possibility that an enhanced response to systemic LPS with prior laparotomy or CORT may be due at least in part to sensitization of peripheral immune cells, specifically peritoneal macrophages. To explore this, we measured the stimulated release of pro-inflammatory products in peritoneal macrophages.

2. METHODS

2.1 Animals

Pathogen-free adult male Sprague-Dawley rats (325–350 g; Harlan Labs, Madison, WI) were used in all experiments. Rats were allowed one week to acclimate to the University of Colorado animal care facility before use. Rats were housed two per cage, with the exception of rats implanted with telemeters for core body temperature and activity, which requires single housing. All rats were housed in temperature (23±3°C) and light (12:12 light: dark; lights on at 07:00) controlled rooms with standard rodent chow and water available *ad libitum*. All procedures were performed during the light phase of the cycle, with the exception of monitoring of core body temperature and activity, which occurred continuously by telemetry without disturbance of the animals in their home cages. The Institutional Animal Care and Use Committee of the University of Colorado at Boulder approved all procedures.

2.2 Surgical procedure

Laparotomy and sham surgeries were performed using aseptic procedures under isoflurane anesthesia (Halocarbon Laboratories, River Edge, NJ) using a previously described method developed as a model of human abdominal exploratory surgery (Martin et al., 2005). The abdominal region was shaved and thoroughly cleaned with 70% ethanol and Exidine-2 surgical scrub. Approximately 0.5 cm below the left, caudal-most rib, a 3 cm diagonal incision was made towards the right, penetrating the peritoneal cavity. Wearing sterile latex gloves, the surgeon inserted their index finger up to the second knuckle into the opening and vigorously manipulated the viscera and musculature. Approximately 10 cm of the intestine were then exteriorized and vigorously rubbed between the surgeon's thumb and index finger for 30 s. The intestines were then placed back into the peritoneal cavity. When thermisters were inserted in laparotomy rats for the monitoring of core body temperature (see below), the probes were introduced through the same abdominal incision and tethered to the abdominal wall after completion of the laparotomy procedure, as previously described (Hansen et al., 2001). Sterile chromic gut sutures (cuticular 4-0, chromic gut, 27", cutting FS-2; Ethicon, Comerville, NJ) were used to suture the peritoneal lining and abdominal muscle in two layers. The skin was closed with surgical staples. To prevent infection, the wound was dressed with Polysporin (Pfizer, Morris Plains, NJ) and 0.25 ml TwinPen (150,000 U/ml penicillin G procaine and penicillin G benzathine; AgrilLabs Ltd., St. Joseph, MO) was administered intramuscularly. Sham-operated rats were anesthetized, shaved and injected with TwinPen and remained on isoflurane for the same amount of time as those that got laparotomy. When thermisters were inserted into sham rats or those for CORT studies, for the monitoring of core body temperature, a small incision was briefly opened to insert and tether the probe to the abdominal wall without manipulation of the gut or musculature. Incision closure and wound treatment was as described above.

2.3 Measurement of core body temperature (CBT) and activity

At the time of laparotomy or sham surgery, calibrated radio transmitters (MiniMitter, Sun River, OR) for measuring CBT were implanted into the peritoneal cavity as described above. CBT and activity was monitored using standard telemetry procedures. For pretreatment with CORT, telemeters were implanted 3 wk before baseline temperature measures were recorded, to prevent effects of the prior surgery on the CORT administration. Briefly, the radiofrequency (Hz) transmitted by the biotelemeter was monitored by a receiver plate (RTA 500, Mini-Mitter, Sunriver, OR, USA) situated beneath the cage of each rat, allowing the rats to move freely and undisturbed in their home cages. The emitted frequency was fed into a peripheral processor (DP-24 DataPort, VitalView, Mini-Mitter, Sunriver, OR, USA) connected to a personal computer and the output expressed in degrees centigrade and activity counts. For activity the telemeter must have moved for it to be counted as activity. Therefore grooming was not counted as activity. Data were collected at one-minute intervals and averaged into one-hour intervals.

2.4 Lipopolysaccharide (LPS) and corticosterone preparation and administration

Lyophilized LPS (25 mg, Escherichia Coli, serotype 0111: B4, Sigma Lot No. 072k4096) was reconstituted with 0.9% endotoxin-free saline (Abbott Laboratories, North Chicago, IL) to derive a 1 mg/ml stock solution that was then aliquoted and stored at −20°C. For *in vivo* application, frozen aliquots were thawed at the time of experimentation and diluted with 0.9% endotoxin-free saline yielding a final concentration of 20 μg/ml. LPS was administered by intraperitoneal injection. For *ex vivo* application, thawed aliquots were serially diluted with culture medium (Iscove's medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and 2 μM L-glutamine; all media reagents from Gibco, Carlsbad, CA). CORT (Sigma, St Louis, MO, USA) was dissolved in propylene glycol (Sigma) at 2.5 mg/ml and administered subcutaneously at the base of the neck at 2.5mg/kg.

2.5 Endotoxin measurement

To define whether prior laparotomy was associated with systemic infection 2 weeks later, endotoxin content was measured in the serum. Samples were slowly thawed on wet ice and centrifuged prior to measurement of gram-negative bacterial endotoxin levels using the limulus amebocyte lysate (LAL) assay (BioWhittaker QCL-1000). The assay was performed according to manufacturer's instructions, the methods of which have been detailed previously (O'Connor et al., 2003). Briefly, samples were diluted (1:250) and mixed with LAL proenzyme. Endotoxin catalyzes the conversion of proenzyme to enzyme. After a brief incubation, substrate was added to each well. Finally, a 25% acetic acid solution was added to each well to stop the enzyme-substrate reaction. Absorbances were measured at 410 nm.

2.6 Nitrite measurement

Nitric oxide (NO) was assessed in culture supernatants by measurement of nitrite (a stable degradative intermediate) as previously described (Fleshner et al., 1998a; Fleshner et al., 1998b). The Greiss reagent used to assay nitrite was prepared immediately before use and consisted of a 1:1 (vol/vol) solution of 1% sulfanilamide (Sigma Chemical, St. Louis, MO) in a 4.25% phosphoric acid solution (Mallinckrodt, St. Louis, MO) and 0.1% N-(1-naphthyl) ethylenediamine dichloride (Sigma Chemical) in distilled-deionized water. A standard curve was generated by making a 2 fold dilution series ranging from 312.5 mM to 305 μM sodium nitrite (Sigma Chemical) in culture medium. Standards and samples were added to 96-well flat-bottom plates (Immulon 4, Dynatech Labs) in duplicates. Griess reagent was added to each well and allowed to incubate at room temperature for 10 min. Absorbances were read at 540-nm wavelength using a microplate reader (model MRX, Dynatech Labs).

2.7 Interleukin-1β protein measurement

Culture supernatants were prepared and assayed following procedures previously described in detail (Hansen et al., 2000; O'Connor et al., 2004). Cell culture supernatants were slowly thawed on wet ice and centrifuged before use. IL-1β protein was measured using commercially available rat-specific enzyme linked immunosorbant assay (ELISA) kits (R&D Systems, Minneapolis, MN) in accordance with manufacturer's instructions. The sensitivity of the assay is 6 pg/ml.

2.8 Data Analysis

All data are presented as mean ± SEM. All statistical comparisons were computed using Graphpad Prism version 5 and consist of either repeated measures, one-way or two-way ANOVA, followed by Bonferroni *post hoc* comparisons. Threshold for statistical significance was set at $\alpha = 0.05$.

2.9 Experimental designs

Experiment 1: Effect of prior CORT on pain responses following LPS—The von Frey behavioral test was performed on the ventral surface of each hind paw within the region of sciatic nerve innervation, as described previously (Milligan et al., 2000). A logarithmic series of 10 calibrated Semmes-Weinstein monofilaments (407 mg-15 136 mg, Stoelting, Wood Dale, IL, USA) was applied randomly to the left and right hind paws, for 8 s at constant pressure. The behavioral testing was performed blind with respect to the drug administration. The stimulus intensity threshold to elicit a paw withdrawal response was used to calculate the 50% paw withdrawal threshold (absolute threshold) using the maximum likelihood fit method to fit a Gaussian integral psychometric function (Harvey, 1986). This method normalizes the withdrawal threshold to parametric conditions. Rats were habituated to the testing environment for 40 min per day on 4 consecutive days. Once baseline measurements were taken, the rats were injected with CORT (2.5mg/kg) or vehicle subcutaneously at the nape of the neck. 24 h following the CORT/vehicle injection, the rats received 20 μg/kg lipopolysaccharide (LPS) or vehicle intraperitoneally ($n = 6$/group). Behavioral testing was done before (BL) and 4 h after CORT/vehicle injection and 4 h after the CORT/vehicle, and before and 1, 2, 4, 6 h and 24 h after LPS or vehicle injection.

Experiment 2: Effect of prior laparotomy on LPS-induced fever and activity— Baseline (BL) CBT was recorded 11 and 12 d after laparotomy or sham surgery ($n = 12$/group) to ensure that normal CBT and circadian rhythms were present prior to the start of the study. As in prior reports (Bilbo et al., 2010), all rats were injected with endotoxin-free saline (equivolume to the LPS dose, below) two days before LPS injection (day 13 post-surgery) to control for the effects of injection on CBT. All rats then received LPS (20 μg/kg, i.p.) 15 d after laparotomy or sham surgery. Following both saline and LPS, CBT was continuously monitored for 48 h. All injections were made within 3 h of lights on.

Experiment 3: Effect of prior CORT on LPS-induced fever and activity— Baseline (BL) CBT was recorded 3 wk after telemeter implant surgery ($n = 12$/group) to ensure that normal CBT and circadian rhythms were present before the start of the study and to ensure that there was no effect of surgery on the experiment. All rats were injected with CORT/vehicle (2.5 mg/kg in propylene glycol, s.c.) followed 24 h by LPS/vehicle (20 μg/kg, i.p.). CBT was monitored 24 h before CORT/vehicle and continued until 48 h after LPS/vehicle. All injections were made within 3 h of lights on.

Experiment 4: Prior laparotomy and prior CORT on LPS-induced nitric oxide and IL-1 in peritoneal cells ex vivo—Peritoneal cells were collected two weeks after

laparotomy or sham surgery (*n* = 3/group) or 24 h after CORT/vehicle (*n* = 3/group). Animals were sacrificed by decapitation and trunk blood (laparotomy only) and peritoneal lavage were collected. Trunk blood was kept on ice and allowed to clot before centrifuging to collect serum (4,000 rpm for 10 min at 4°C). Serum was stored at −20°C until time of assay. Peritoneal cells (shown previously by our laboratory using this procedure to be predominately macrophages (Fleshner et al., 1998a) were collected by peritoneal lavage. Thirty ml of ice-cold Hank's balanced salt solution was injected into the abdominal cavity, the abdomen massaged for 30 s after which the fluid was removed (~20 ml) and centrifuged at 1000 rpm for 5 min at 4°C. The cells were treated with red blood cell lysis buffer (160 mM NH_4Cl, 12 mM $NaHCO_3$, 100 μM EDTA, dissolved in dH_2O, pH 7.3) for 2 min. The cells were resuspended in 20 ml Iscove's medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and 2 μM L-glutamine was added and centrifuged at 1,000 rpm for 5 min. Cells were resuspended in media and plated at 200,000 cells per well/ 200 μl media in a 96-well tissue culture plate. 2 h after plating the cells were washed with warm Dulbecco's phosphate buffered saline, to remove any non-adherent cells and resuspended in 200 μl media. The cells were cultured for 24 h *ex vivo* with 0, 1, 10 and 100 ng/ml LPS at 37°C with 5% CO_2. Following incubation cells were centrifuged and supernatants were collected. A portion of the supernatant was immediately assessed for NO production and the remainder was stored at −20°C until assay for IL-1β levels.

3. RESULTS

3.1 Endotoxin concentration following laparotomy

Serum concentrations of endotoxin were unchanged 2 wk after laparotomy ($F_{1,99} = 0.12$, $P > 0.05$), suggesting there were no surgery-related gram negative bacterial infections at this time point.

3.2 Prior CORT interacts with sub-threshold LPS to cause allodynia

We have shown previously that prior laparotomy is able to sensitize the immune system to subsequent LPS challenge resulting in mechanical allodynia (Hains et al., 2010). In addition, we have shown that CORT pretreatment exaggerates the immune response to a subsequent challenge by spinal administration of LPS (Loram et al., 2011). However, no prior study has demonstrated whether CORT sensitization of subsequent glial/immune responses is sufficient to result in observable behavioral changes, when the subsequent immune challenge is systemic. Therefore, we assessed the effect of CORT and subsequent systemic LPS on pain thresholds assessed on the hind paws. The dose of LPS (20 μg/kg) used in this study is on the low side of those reported to induce robust fever (Kapas et al., 1998). We chose this relatively low dose of LPS so to allow for detection of a potentiated response, were one to occur. Figure 1 shows the behavioral response to mechanical stimulation applied to the hind paw before systemic CORT or vehicle and before and after systemic LPS or vehicle. 20 μg/kg of LPS (i.p.) alone did not induce significant allodynia compared to vehicle controls (post-hoc, $P>0.05$). However, when systemic CORT was given 24 h before LPS, there was significant allodynia from 1–4 h after LPS (Interaction: $F_{27,156}=3.09$, $P<0.0001$) compared to CORT+vehicle and vehicle+vehicle control ($P<0.01$) and 1 and 2 h after LPS compared to vehicle+LPS ($P<0.001$). Therefore, prior CORT sensitizes the system such that allodynia is induced by a mild dose of LPS that given alone would not cause significant pain responses.

3.4 Prior laparotomy potentiates LPS-induced fever

We have previously shown that prior laparotomy enhances LPS-induced pain responses (Hains et al., 2010). In the current study we tested whether the effects of laparotomy may generalize to sickness responses beyond pain. Here, we examined a classic and well-

characterized sickness response, namely LPS-induced fever. Regardless of prior surgical treatment, all animals showed normal circadian rhythm of body temperature when CBT recordings began 11 d after surgery. Injection and handling on the vehicle injection day (13 d after surgery) and the LPS injection day (15 d after surgery) caused a brief stress fever that was not influenced by prior laparotomy (Fig. 2). Compared to vehicle injection, LPS resulted in a time-dependent fever response only in rats with prior laparotomy. Repeated measures ANOVA on CBT in the light phase following resolution of stress hyperthermia (11:00 – 19:00) revealed that there was a significant interaction between injection and time ($F_{24,328} = 2.12$, $P < 0.001$) with significant differences between LAP+vehicle and LAP+LPS from 14:00–16:00 (P<0.01), and sham+vehicle and LAP+LPS from 13:00–17:00 (P<0.01) and between LAP+LPS and sham+LPS at 15:00 (P<0.01).

3.5 Prior CORT potentiates LPS-induced fever

Before any injections, the rats displayed normal circadian rhythm. CORT had no significant effect on body temperature for the 24 h after CORT/vehicle admin before LPS administration. CBT in the light phase following resolution of stress hyperthermia (11:00 – 19:00) revealed that there was a significant interaction between injection and time ($F_{24,300} = 2.64$, $P < 0.0001$) with significant differences between CORT+LPS and vehicle+vehicle from 1100–1600 (P<0.001) and between CORT+LPS and vehicle+LPS from 13:00–15:00 (P<0.01).

3.6 LPS creates sickness induced lethargy that is not potentiated by prior laparotomy or prior CORT

In order to determine whether prior laparotomy or CORT are also able to potentiate sickness-induced lethargy, activity was analyzed in hourly increments in the active dark phase. Activity counts were summed per hour and normalized to percent of baseline, where the baseline values were collected the night before any injections. The first 6 h after lights off were used in the analysis, as there were no significant differences between groups in the second half of the dark phase. The summed hourly activity counts within the first 6 h after lights off were averaged and presented in Figure 4. For activity following LPS or vehicle in LAP/sham rats, there was a significant main effect of LPS ($F_{1,1}$=5.89, P<0.05, Fig. 4A), with LPS suppressing the activity. There was no significant main effect of surgery ($F_{1,1}$=0.22, P=0.65) or significant interaction ($F_{1,40}$=0.11, P=0.75). While surgery did not affect activity measures it was associated with a significant reduction in weight gained over the 14 d after laparotomy (data not shown; 25.38 g ± 1.96 g, mean ± SEM) compared to sham surgery (32.79 g ± 2.16 g; $F_{1,108} = 6.37$, $P < 0.025$). In contrast, CORT did not affect body weight within the time frame of the current experiments.

There was no significant effect of CORT on activity at any time of the dark/active phase of the light cycle (measured from lights off to lights on, data not shown). In the first 6 h of the dark phase following LPS/vehicle in rats receiving CORT/vehicle, there was a significant main effect of LPS ($F_{1,1}$=22.79, P<0.0001, Fig. 4B), with LPS suppressing the activity. There was no significant main effect of CORT ($F_{1,1}$=0.79, P=0.38) or interaction ($F_{1,40}$=1.84, P=0.19).

3.7 Prior laparotomy but not prior CORT potentiates LPS-induced nitric oxide (NO) responses in peritoneal cells ex vivo, with no effect on IL-1β

Ex vivo LPS induced NO release and IL-1β release was measured in peritoneal cells harvested 2 wk after laparotomy or sham surgery, or 24 h after CORT/vehicle (Fig. 5). *Ex vivo* stimulation of peritoneal cells with LPS produced dose-dependent increases in nitrite concentration measured in supernatants of cells collected from laparotomy and sham rats 24 h after LPS (Fig. 5A). Nitrite responses to LPS doses were greatly enhanced by previous

laparotomy (Interaction: $F_{3,24}$=9.44, $P < 0.01$) with prior laparotomy resulting in higher nitrite responses at all doses of LPS (P<0.001). There was no significant effect of surgery on IL-1β expression but there was a significant LPS dose response ($F_{3,6}$=25.62, P<0.001, Fig. 5C). In peritoneal cells collected 24 h after CORT treatment there was a significant LPS dose response for both nitric oxide ($F_{3,24}$=28.65, P<0.0001, Fig. 5B) and IL-1β ($F_{3,6}$=25.62, P<0.001, Fig. 5D) but no significant CORT effect or interaction.

4. DISCUSSION

The present series of studies extend existing knowledge by demonstrating that two stressors, CORT and laparotomy, similarly affected the responses to subsequent low dose systemic LPS: both potentiated sickness-induced fever and pain but not lethargy. Interestingly, prior laparotomy, but nor prior CORT, produced a potentiated production of nitric oxide in peritoneal macrophages when stimulated with LPS *ex vivo.* Neither pretreatment affected LPS-induced IL-1β release *ex vivo*.

Potentiation of the fever response has been shown previously with a “two-hit” paradigm, inescapable tail shock, 24 h before low dose LPS (O’Connor et al., 2003). An acute series of inescapable tail shocks causes the release of glucocorticoids equivalent to the dose of exogenous CORT administered in our studies (O’Connor et al., 2003). Both inescapable shock and exogenous CORT potentiate the LPS-induced proinflammatory cytokine production peripherally (Frank et al., 2010; Johnson et al., 2002; O’Connor et al., 2003). Therefore, it is not surprising that prior CORT produced a potentiated fever comparable to that seen after inescapable shock, but rather clarifies that CORT is a key mediator in the potentiated fever response following an acute stressor.

Interestingly, laparotomy was done 2 wk before LPS administration, a much longer duration before LPS than prior CORT or an acute stressor, and yet the potentiated responses were still observed with the same amplitude of fever and of a similar duration. This effect of potentiated inflammatory response was not seen with a 4 wk delay after laparotomy (Hains et al., 2010). Therefore, it will be intriguing to define whether a similarly prolonged critical window exists for tail shock, glucocorticoids or other challenges.

We have previously shown that laparotomy 2 wk before low dose systemic LPS administration potentiated sickness-induced pain responses (allodynia). Therefore, we explored not only the classic fever response but also other behaviors such as sickness-induced pain by prior CORT. Prior stress, prior CORT and prior laparotomy each potentiate sickness-induced or injury-induced allodynia (Alexander et al., 2009; Hains et al., 2010; Takasaki et al., 2005). Glia within the spinal cord produce proinflammatory cytokines that potentiate the allodynia (Watkins, 2005). Spinal microglia are activated after laparotomy and enhancement of allodynia due to prior laparotomy is suppressed by intrathecal administration of the microglial inhibitor, minocycline (Hains et al., 2010). With regards to sensitization by CORT, enhancement of allodynia to LPS administered into the spinal cord is abolished by intrathecal interleukin 1 receptor antagonist (Loram et al., 2011). Therefore, spinal microglia likely have a role to play in this pain potentiation.

While there are common mechanisms inducing the sickness responses such as lethargy and fever, there is some suggestion that certain sickness responses are more responsive to some released pro-inflammatory cytokines than others (Harden et al.). Lethargy is a clinical symptom of infection and is identified in animal models by measuring either voluntary cage activity or voluntary running wheel activity. In both the CORT and laparotomy studies, there was a significant lethargy induced within the first 6 h of the dark phase by LPS when it was administered within the first 4 h after lights on. However, neither challenge enhanced the

effect of LPS on lethargy. It is possible that if LPS was administered within the dark phase of the cycle that a greater suppressive effect would have been identified. However, activity following LPS, with a prior inescapable shock showed a main effect of LPS and a main effect of shock, irrespective of whether the LPS was administered at the beginning of the light phase or at the beginning of the dark phase (Johnson et al., 2003). While there was a trend of further suppression of activity by prior CORT in our study, it was not significant.

Previous work also has shown that the lethargy following systemic LPS outlasted the effects of the fever (Skinner et al., 2009). We tested on the second day and the subsequent night and found no delayed effects of either laparotomy or CORT on the activity (data not shown). It is possible that voluntary wheel running may have been a more sensitive measure of activity compared to voluntary cage activity. While both measures were suppressed following two different doses of LPS, the wheel running was a more sensitive and showed a more robust suppression (Hopwood et al., 2009). Therefore, it is possible that sickness-induced lethargy is not as sensitive to the effects of prior surgery and prior CORT as compared to fever and allodynia.

Most studies investigate the effect of infection on fever or tissue response with only a few studies examining the effect on both fever and other sickness-induced behaviors including lethargy. Blocking peripheral IL-6 partially attenuated the lethargy induced by LPS but completely blocked the fever (Harden et al., 2006). In addition, diclofenac, a cyclooxygenase inhibitor blocking the synthesis of PGE2, only partially blocked LPS induced lethargy but completely blocked the fever (Harden et al., 2010). Therefore, it is possible that laparotomy and CORT sensitize pathways contributing predominantly to fever and sickness-induced allodynia and less to the pathways inducing lethargy.

A pathogen-host interaction where immune cells release IL-1, NO and other pro-inflammatory substances initiate the sickness-induced pain and fever pathways. Peripheral to central signaling triggers immune cells in the brain and spinal cord to also release pro-inflammatory cytokines. Both the peripheral and central immune cells involved in the sickness response can become sensitized. Cellular sensitization is defined as the effect of an initial insult on a cell's response to a subsequent insult. Sensitized cells are hypervigiliant, but do not exhibit an activated phenotype. Thus, while they respond to subthreshold stimuli and exhibit an exaggerated response to suprathreshold stimuli, they are not producing proinflammatory cytokines or engaging in respiratory burst activity (Ma et al., 2003).

Laparotomy involves both tissue damage and an introduction of enteric bacteria into the peritoneal cavity, and thus there is an activation of local immune cells in the period immediately following surgery with an increase in pro-inflammatory cytokines (Kim and Yoon, 2010; Lee et al., 2003). It is possible that peritoneal macrophages can become sensitized as an effect of this initial immune response. Peritoneal cells stimulated within 24 h after surgery display decreased phagocytic activity, decreased TNFα release, and reduced endotoxin clearance (Pitombo, 2006; Moehrlen, 2005). However, we observed enhanced NO release to LPS in these cells two weeks post-surgery. Although no effect was found on IL-1 release to *ex vivo* LPS, it is possible that IL-1 was produced intracellularly but not released from cells under the experimental cell culture conditions. Increased IL-1 mRNA was in fact noted in unstimulated peritoneal cells collected 2 wk after laparotomy compared to sham surgery (Hains, Unpub. Observ.).

The current finding that CORT did not affect peritoneal macrophage responses is consistent with previous studies. Even when administered *in vitro*, pretreatment with CORT caused a significant reduction in TNF secretion following LPS stimulation (Du et al., 2010). Unlike laparotomy, CORT treatment does not cause inflammation or local immune activation. It is

more likely that the effect of prior CORT on fever and pain is from an enhanced CNS immune response, involving sensitized microglia. Laparotomy may also lead to microglial sensitization (Hains et al., 2010), in addition to the effect on peritoneal macrophages, as demonstrated here.

Along with perivascular and meningeal macrophages, microglia represent a primary source for IL-1 in the CNS (van Dam et al., 1992). Sensitized microglia are characterized by exaggerated IL-1 production in response to stimulation. Our group has recently shown that both stress and CORT caused increased IL-1 production in microglia following *ex vivo* LPS (Frank et al., 2007; Frank et al., 2010). In addition, microglial activation within the spinal cord is noted 2 wk after laparotomy (Hains et al., 2010), suggesting that microglia were not quescient at the time of LPS injection in the present studies. While microglial phenotype was not examined here and no conclusions can be made from the current findings regarding the role of microglia in sensitized sickness responses, it certainly warrants further investigation.

An immune system sensitized by prior surgery or prior CORT results in potentiation of some but not all potentiated sickness-induced behaviors. While the two challenges under investigation are distinct in their site and underlying mechanism, the potentiation of the fever and pain induced by low dose LPS are comparable. This is despite the fact that laparotomy was done 2 wk before the LPS while CORT was administered 24 h before the LPS. Through immune-to-brain-to-spinal cord communication, systemic LPS activates immune cells both in the periphery and within the central nervous system. During infection, there is a release by peripheral immune cells of pro-inflammatory cytokines and other proinflammatory mediators such as nitric oxide and reactive oxygen species. The communication of these peripheral immune signals to the brain and spinal cord result in fever and other sickness behaviors. While laparotomy induces an injury and subsequent inflammation, CORT alone does not elevate proinflammatory cytokines, but rather is classically anti-inflammatory and suppresses inflammatory responses when administered in the presence of inflammation (Sorrells et al., 2009). Therefore, while CORT must sensitize the immune system, as demonstrated by work from our lab and others (Frank et al., 2010; Munhoz et al., 2006) such that a subsequent immune challenge results in exaggerated sickness behaviors including pain, it must do so in a different manner to that of prior surgery.

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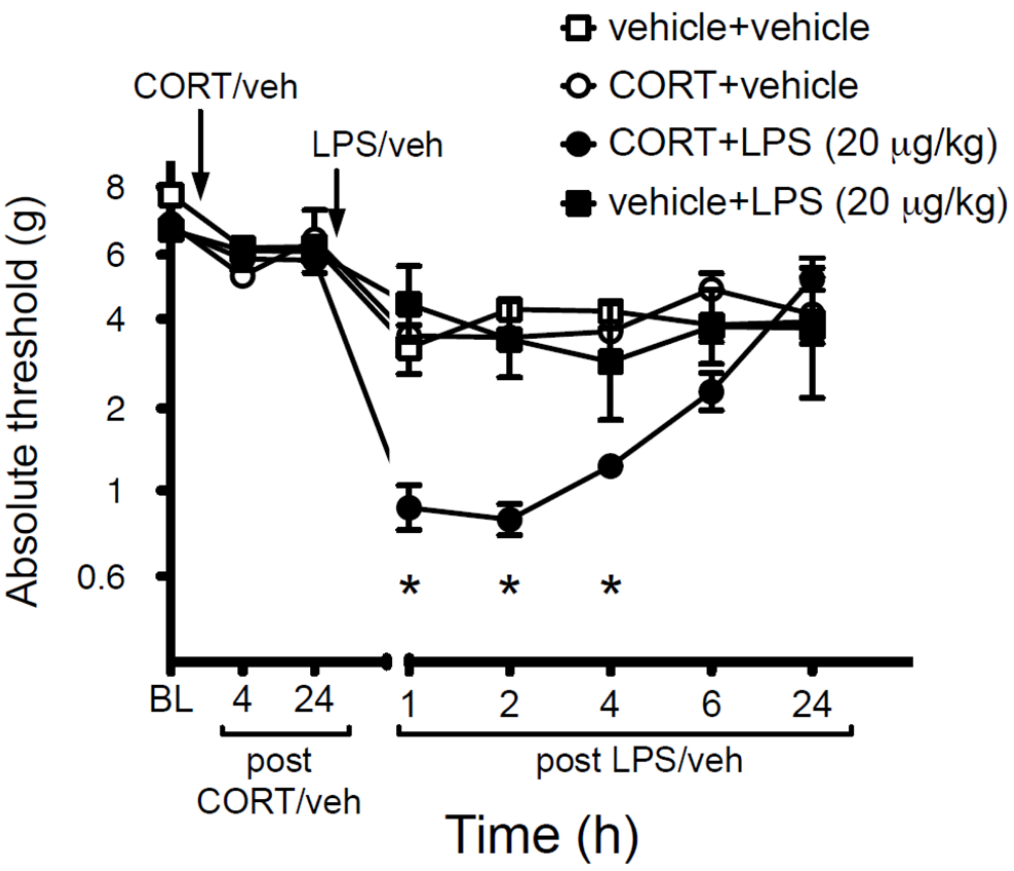

Figure 1.

Systemic administration of 20 μg/kg (i.p.) LPS induces no significant allodynia alone. Prior administration of CORT (2.5 mg/kg, s.c.), given 24 h before LPS, induced significant allodynia. Mechanical sensitivity was tested using the von Frey test, before (baseline; BL) CORT or vehicle, before (0) and 1–24 h after i.p. LPS or equivolume vehicle. Solid circle depicts CORT+LPS (n = 6), solid squares depict vehicle+LPS (n=6), open circles depict CORT+vehicle (n=6) and open squares depict vehicle+vehicle (n = 6). Data are presented as average absolute thresholds of left and right hind paws (mean ± SEM). *P < 0.001, CORT +LPS against CORT+vehicle and vehicle+vehicle at the same time point. # P<0.001 CORT +LPS against vehicle+LPS at the same time point.

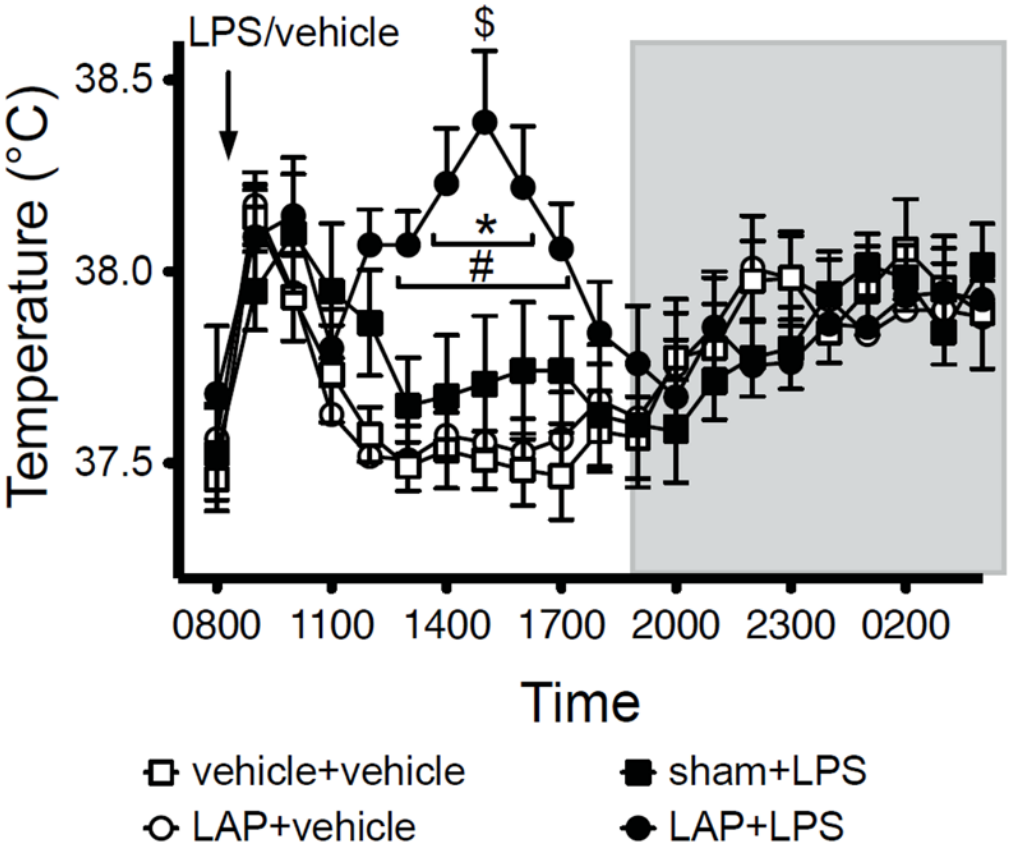

Figure 2.

Systemic administration of LPS (20 μg/kg, i.p.) caused elevated CBT only when preceded by laparotomy (LAP), performed 2 wk prior. LAP produced a significantly greater increase in CBT 4 to 6 h after LPS injection compared to sham treatment. Values represent averaged CBT across one-hour bins ± SEM. Solid circle depicts LAP+LPS (n=11), solid squares depict Sham+LPS (n=12), open circles depict LAP+vehicle (n=12) and open squares depict Sham+vehicle (n = 12). * $P < 0.01$ against LAP+vehicle, # $P<0.01$ against sham+vehicle, $ $P<0.01$ against sham+LPS.

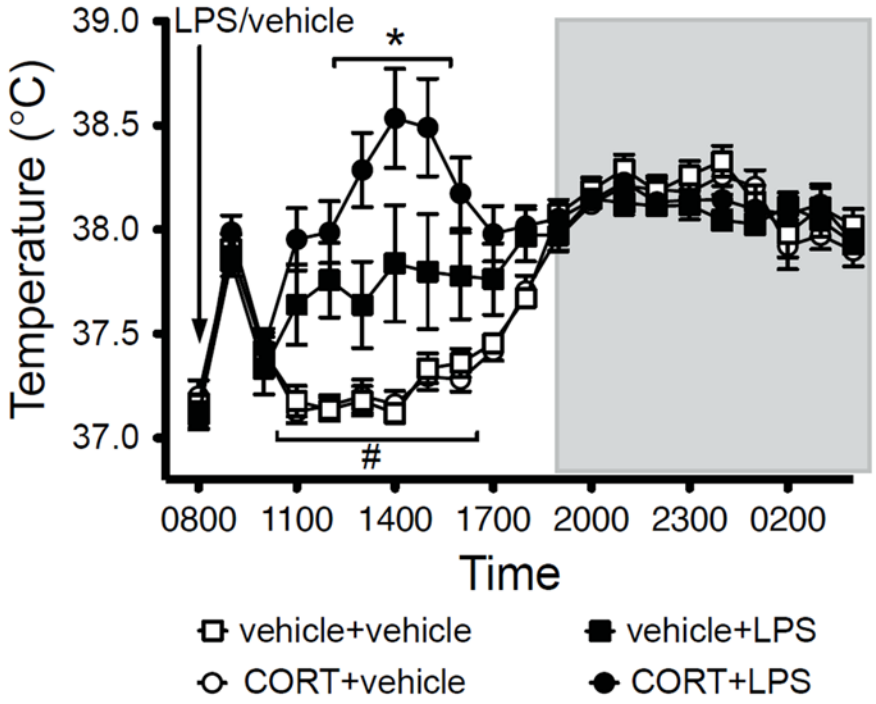

Figure 3.

Hyperthermia induced by i.p. LPS is greatly enhanced by administration of CORT 24 h prior. Values represent averaged CBT across one-hour bins ± SEM (n=10 per group). CORT produced a significantly greater increase in CBT 4 to 6 h after LPS injection compared to vehicle treatment. Solid circle depicts CORT+LPS, solid squares depict vehicle+LPS, open circles depict CORT+vehicle and open squares depict vehicle+vehicle. * P<0.001 CORT +LPS against vehicle+LPS, # P<0.001 CORT+LPS against vehicle+vehicle.

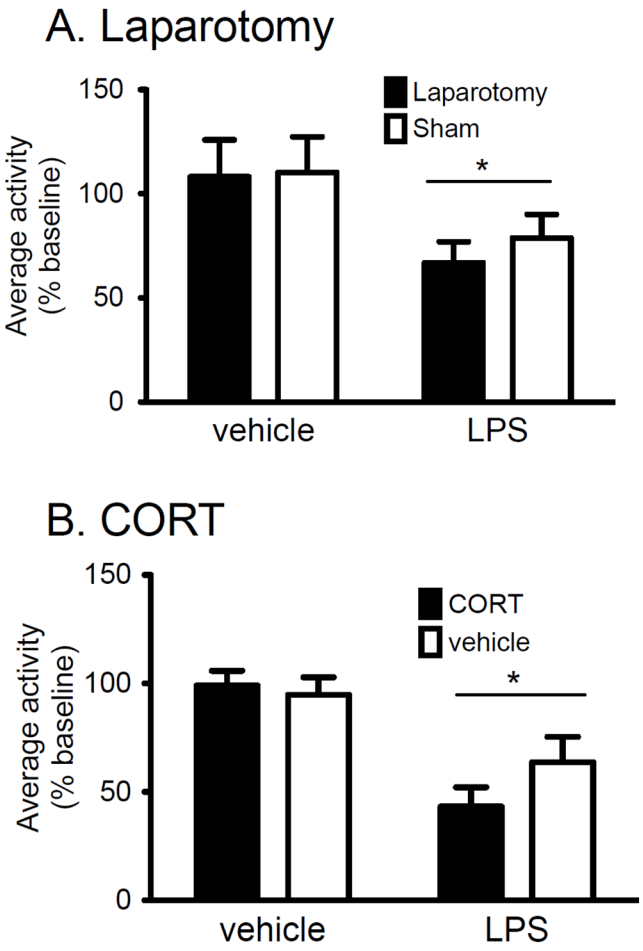

Figure 4.

Neither laparotomy (Panel A) and nor CORT (Panel B) influenced suppression of spontaneous cage activity due to i.p. LPS administered 2 wk after surgery or 24 h after CORT. Values represent averaged activity from the first 6 h of lights off where activity counts were summed into 1 h bins (means ± SEM, n=10–12 per group). There was a significant LPS effect for both groups but no main effect of LAP or CORT.

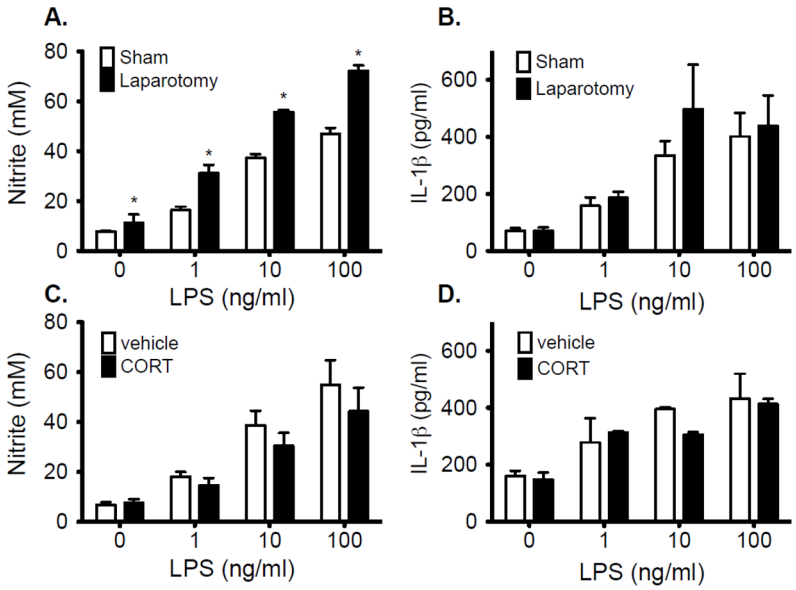

Figure 5.

Laparotomy enhanced the stimulated release of NO (as measured by nitrite; Panel A) but not IL-1 (Panel B) from peritoneal cells while CORT had no effect on either NO (Panel C) or IL-1 (Panel D) levels. Peritoneal cells were harvested 2 wk after laparotomy and 24 h after CORT and stimulated *ex vivo* with with LPS (0, 1, 10 and 100 ng/ml) for 24 h. LPS dose-dependently increased nitrite and IL-1 levels in supernatants of all cell samples. Data are presented as percentage of sham + vehicle (means ± SEM). Each condition was plated in triplicate and n=3/4 rats per group. *P < 0.05 compared to sham control at each dose of LPS.