Increased cytokine and chemokine gene expression in the CNS of mice
during heat stroke recovery

Joseph C. Biedenkapp¹
Lisa R. Leon²

US Army Research Institute of Environmental Medicine
¹Military Nutrition Division
²Thermal and Mountain Medicine Division
Natick, Massachusetts 01760-5007

Running Title: CNS gene expression changes during heat stroke recovery

Address Correspondence To:
Lisa R. Leon
US Army Research Institute of Environmental Medicine
Thermal and Mountain Medicine Division
Kansas Street, Building 42
Natick, Massachusetts 01760-5007
Phone: 508-233-4862
Fax: 508-233-5298
Email: lisa.r.leon.civ@mail.mil
Abstract:

Heat stroke (HS) is characterized by a systemic inflammatory response syndrome (SIRS) consisting of profound core temperature (T_c) changes in mice. Encephalopathy is common at HS collapse, but inflammatory changes occurring in the brain during the SIRS remain unidentified. We determined the association between inflammatory gene expression changes in the brain with T_c disturbances during HS recovery in mice. Gene expression changes of heat shock protein (HSP)72, heme-oxygenase (hmox1), cytokines (IL-1β, IL-6, TNFα), cyclooxygenase enzymes (COX-1, COX-2), chemokines (MCP-1, MIP-1α, MIP-1β, CX3CR1), and glia activation markers (CD14, aif1, vimentin) were examined in the hypothalamus (HY) and hippocampus (HC) of control (T_c~36.0°C) and HS mice at T_c,Max (42.7°C), hypothermia depth (HD; 29.3±0.4°C) and fever (37.8±0.3°C). HSP72 (HY<HC) and IL-1β (HY only) were the only genes that showed increased expression at T_c,Max. HSP72 (HY<HC), hmox1 (HY<HC), cytokine (HY=HC), and chemokine (HY=HC) expression was highest at HD and similar to controls during fever. COX-1 expression was unaffected by HS, whereas HD was associated with ~3-fold increase in COX-2 expression (HY only). COX-2 expression was not increased during fever and indomethacin (COX inhibitor) had no effect on this T_c response indicating fever is regulated by other inflammatory pathways. CD14, aif1, and vimentin activation at HD coincided with maximal cytokine and chemokine expression suggesting glia cells are a possible source of brain cytokines and chemokines during HS recovery. The inflammatory gene expression changes during HS recovery suggest cytokines and/or chemokines may be initiating development or re-warming from hypothermia whereas fever pathway(s) remain to be elucidated.

Key Words: heat stroke; cytokines; chemokines; hypothermia; fever; systemic inflammatory response
Introduction:

Heat stroke (HS) is a serious form of hyperthermia characterized by a systemic inflammatory response syndrome (SIRS) leading to multi-organ dysfunction in which encephalopathy (e.g., ataxia, delirium, coma) predominates (5). The most common central nervous system (CNS) disturbance in HS patients is cerebellar dysfunction, which manifests as ataxia at the time of collapse (5, 25, 26). Postmortem analysis of HS victims showed histological damage and glia proliferation in the cerebellum that appeared to be the pathologic basis for motor sequelae observed upon clinical admission (25). However, not all pathophysiological responses associated with the SIRS show a direct correlate to brain regions that experience cellular damage with HS. For example, HS patients and animal models often display core temperature ($T_c$) disturbances (e.g., hypothermia, fever) during recovery that occur in the absence of damage to the pre-optic anterior hypothalamus (POAH), which is considered the main thermoregulatory center in the brain (1, 21, 25). Since many of the thermoregulatory and sickness symptoms of HS are transient in nature, they are likely due to inflammatory changes rather than heat cytotoxicity and brain damage *per se*.

The central and systemic mechanisms mediating the SIRS to HS have not been fully elucidated, but circulating cytokines (e.g., interleukin [IL]-1β, IL-6, tumor necrosis factor [TNF]α) and chemokines (monocyte chemoattractant protein-1 [MCP-1]) have been implicated in the morbidity of this syndrome (6, 19, 23). High circulating IL-6 showed the highest correlation with neurological symptoms and morbidity of HS patients and animal models (4, 6, 19). Cytokines act within the CNS to mediate $T_c$ and sickness responses to peripheral inflammation (e.g., lipopolysaccharide [LPS], a bacterial cell wall component), but it remains unknown if similar actions are occurring during the SIRS to HS (16). Bidirectional
communication between the peripheral immune system and the CNS may be a mechanism by which resident brain cells are stimulated to produce cytokines, chemokines and other inflammatory mediators of the SIRS symptoms (11). Glia are considered the main producers of cytokines and chemokines during peripheral inflammation (33) and may be operating similarly during HS (9, 31). Yet, the association between glial reactivity, cytokine and/or chemokine production in the brain and heat-induced SIRS responses has not been investigated. IL-1β is one of the first cytokines observed during the early stages of the SIRS and may increase the production of other cytokines, such as IL-6 and TNFα. IL-1β was increased in the hypothalamus of rabbits at the onset of HS, but the association of this response with other cytokines or inflammatory mediators in this brain region was not investigated. Furthermore, anesthesia increased IL-1β in the absence of heat exposure complicating interpretation of this finding (22).

The goal of the current study was to exploit a well-characterized conscious mouse model to determine the association between inflammatory gene expression changes occurring in the brain with Tc disturbances and sickness behaviors displayed during 24-h of HS recovery. We quantified the profile of gene expression changes in heat shock proteins (HSPs), glial reactivity markers, cytokines, chemokines and cyclooxygenase (COX) enzymes and compared these responses with the pattern of Tc changes (hyperthermia, hypothermia and fever) and lethargy (decreased home-cage activity) observed during HS recovery. Responses were compared between the hypothalamus and hippocampus, which were considered the main thermoregulatory and stress axis integration sites in our mouse HS model, respectively. Three hypotheses were tested in this study: (1) The hippocampus and hypothalamus will show a differential profile of gene expression changes indicating regional specificity in the response to hyperthermia rather than a generalized CNS response. (2) Tc disturbances and lethargy during the SIRS will be
associated with increased cytokine (IL-1β, IL-6, TNFα) and COX gene expression changes, suggesting regulation by these pathways in the brain. (3) The fever response observed during the day after heat exposure is a regulated response to an increase in the Tc balance point (or set point) that is mediated by cytokine activation of COX enzymes. To test this last hypothesis, we examined the effect of indomethacin (a non-steroidal anti-inflammatory drug [NSAID] that inhibits COX enzymes) on the fever response to HS.

Methods:

Animals. Male C57BL/6J male mice weighing 25.3 ± 0.6 g were individually housed in Nalgene polycarbonate cages (11.5 in x 7.5 in x 5 in) fitted with HEPA-filter cage tops and Alpha-Dri bedding (PharMase, Framingham, MA). Rodent laboratory chow (LM-485; Harlan Teklad, Madison, WI) and water were provided ad libitum under standard environmental conditions (25 ± 2°C; 12:12-h light-dark cycle, lights on at 0600). In conducting research using animals, we adhered to the Guide for the Care and Use of Laboratory Animals in an Association for Assessment and Accreditation of Laboratory Animal Care accredited facility. All procedures received Institutional Animal Care and Use Committee approval before experimentation.

Radiotelemetry. Mice were intraperitoneally implanted with a battery-operated, free-floating radiotelemetry transmitter device (gene expression and home-cage activity analyses; Model TA10TA-F20; Data Sciences International, St. Paul, MN) or a battery-free device (fever experiment; model G2 Emitter, Mini Mitter Co., Inc., Bend, OR) for remote sensing of Tc (±0.1°C) and activity (counts). The transmitters emitted a unique frequency (Hz) that was proportional to Tc and detected by an antenna under each animal's cage; frequency values were transferred to a
peripheral PC and converted to $T_c$ using predetermined calibration values. Calibration values were verified prior to and following experimentation to ensure accuracy of $T_c$ measurements.

Home-cage activity was used as a measure of lethargy and was monitored in a separate group of mice than those used for gene expression analysis. Activity was detected as changes in signal strength with animal movement on the receiver board representing a general measure that did not distinguish between motor movements and postural changes. $T_c$ and activity were remotely monitored at 1-min intervals in conscious unrestrained mice throughout experimentation.

---

*Surgery.* Mice were anesthetized with isoflurane anesthesia, the abdominal fur was shaved and the area scrubbed with a 10% Povidone-iodine solution (Betadine Solution, Purdue Frederick, Stamford, CT) followed by 70% isopropyl alcohol. Each radiotelemetry device was disinfected by pre-soaking for 1h in cold sterilant (Actril®, Minntech Corporation, Minneapolis, MN) followed by three rinses in 0.9% sterile saline. A 1-cm incision was made through the skin and abdominal muscle layer using aseptic technique. Rinsate was tested to ensure that no residual Actril® was present (<10 ppm). The peritoneal muscle and skin layers were closed with absorbable suture (5–0 Coated Vicryl, RB-1 Taper; Ethicon, Somerville, NJ) using interrupted and continuous subcuticular patterns, respectively. Immediately following surgery, each mouse was placed into a clean cage with *ad libitum* food and water and returned to the animal room for undisturbed recovery. Mice were provided ibuprofen analgesia (Children’s Advil, Cold Formula, grape, Wyeth Healthcare, El Paso, TX) in the drinking water (200 $\mu$g/ml) 24h prior to surgery and by injection (30 mg/kg, sc) immediately following surgery. To avoid any confounding influence of ibuprofen analgesia (an NSAID) on the fever experiment, the mice used in this experiment
were provided analgesia as a subcutaneous injection of buprenorphine hydrochloride (Henry Schein, Melville, NY; 0.05 mg/kg) immediately prior to transmitter surgery and on days 1 and 2 of recovery. Experimentation was not begun until surgical recovery was achieved, as defined a priori as a return to pre-surgical body weight, food and water intake, as well as stable circadian T<sub>c</sub> and activity rhythms (typically ≥ 1 week; (19).

Heat stress protocol. The heat stress protocol has been described in detail elsewhere (20). Briefly, ~24h prior to heat exposure, mice in their home cages with food, water, and bedding were placed into a floor-standing incubator (Therma Forma, Model 3950, Marietta, OH) to acclimate to the fan noises at the normal housing temperature of 25 ± 2°C. Cage filter tops were removed to facilitate air circulation. The following day between 0800 and 1000h, mice with T<sub>c</sub><36.0°C (typical daytime, inactive T<sub>c</sub> for this species) were weighed, food and water were removed from the cage and the incubator ambient temperature (T<sub>a</sub>) increased to 39.5 ± 0.2°C (required ~1h to reach this T<sub>a</sub>). Mice remained in the heat stress environment until T<sub>c</sub>,Max of 42.7°C (gene expression and home-cage activity analyses) or 42.4°C (fever analysis) was reached. At T<sub>c</sub>,Max, mice were removed from the heat, body weight was recorded and food and water placed back into the cage for undisturbed recovery at 25 ± 2°C.

Brain harvesting and micropunches: Brain samples were from a previous study with the T<sub>c</sub> responses reported elsewhere and provided here as a group descriptor only (19). Briefly, HS mice were randomly assigned to one of the following groups for brain harvesting: (I) baseline (immediately prior to heat exposure; n=6 [T<sub>c</sub>=35.7 ± 0.1°C]), (II) T<sub>c</sub>,Max (control n = 7 [T<sub>c</sub>=35.6 ± 0.2°C], heat n = 7 [T<sub>c</sub>=42.7 ± 0.0°C]), (III) hypothermia depth (HD; lowest 1-min T<sub>c</sub> value
during recovery with 0.01°C cooling rate; control $n = 5$ [$T_c=36.4 \pm 0.4^\circ C$], heat $n = 7$ [$T_c=29.3 \pm 0.4^\circ C$], (IV) fever (24h following the start of heat exposure; control $n = 6$ [$T_c=36.2 \pm 0.3^\circ C$], heat $n = 6$ [$T_c=37.8 \pm 0.3^\circ C$]). Control mice were exposed to the same conditions at $T_a$ of 25 ±

2°C with the timing of experimental procedures matched to that of a heat-exposed mouse. Control groups were analyzed at each time point to account for any circadian influences on gene expression.

The organ harvesting procedures used in this study were reported elsewhere (19). Briefly, mice were rapidly anesthetized (< 1 min) with isoflurane and exsanguinated following thoracotomy and intracardiac puncture (heparinized 1 ml syringe, 23 gauge needle). Mice were infused with sterile saline and the brain rapidly excised, frozen in liquid nitrogen, and stored at -80°C until analysis. Each frozen brain was mounted in a cryostat and 1mm bilateral micropunches were obtained from the hypothalamus and dorsal hippocampus using a brain punch set (Brain Punch Set, MyNeurolab, Buffalo Grove, IL). Micropunches were transferred to a 1.5ml microcentrifuge tube on dry ice containing homogenization beads (Matrix D, MP Biomedicals, Solon, OH).

Real-time PCR. Hypothalamus and hippocampus micropunches were homogenized in RNA Lysis Buffer using the Fast-Prep 24 (MP Biomedicals, Solon, OH) and RNAqueous Micro Kit (Ambion, Grand Island, NY) following vendor instructions. RNA concentration and quality were determined using a Nanodrop 8000 spectrophotometer (Nanodrop Products,Wilmington, DE). RNA concentration was calculated from the 260 nm reading. RNA purity for all samples used in real-time PCR analysis was >1.8 for the 260/280 nm ratios.
RNA samples were reverse transcribed into cDNA using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA) per vendor instructions. Samples were reverse transcribed at 37°C for 2h, the resultant cDNA was used in real-time PCR experiments. TaqMan Expression Assays containing 0.9 µM of each primer and 0.25 µM of a TaqMan MGB probe, comprising an FAM reporter dye at the 5’-end and a nonfluorescent quencher at the 3’-end, were combined with 12.5 ng cDNA and water in a final reaction volume of 20 µl.

Primers and probe sets for HSP72, heme-oxygenase 1 (hmox1), murine IL-1β, IL-6, TNF-α, COX-1, prostaglandin-endoperoxide synthase 2 (Ptgs2; COX-2), CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CX3CR1, CD14, toll-like receptor (TLR)4, allograft inflammatory factor 1 (Aif1), vimentin, and 18s RNA were obtained from Applied Biosystems.

Amplification of PCR products was performed under the following conditions: 10 min at 95°C followed by 40 cycles consisting of 95°C for 15 s and 1 min at 60°C. The threshold cycle (Ct) for each gene was defined as the PCR cycle at which the emitted fluorescence signal was greater than the background level of fluorescence. For ease of presentation, all genes are referred to by common names, which are listed in Table 1 along with gene nomenclature and function.

Changes in target gene expression within the hypothalamus and hippocampus were calculated as fold-change relative to non-heated controls at the same time point (TcMax, hypothermia, fever) using the $2^{\Delta\Delta Ct}$ method. Data were normalized, denoted as the ΔCt, by determining differences in Ct values between the target gene of interest and the 18s internal control gene. Mean normalized Ct values at baseline, TcMax, hypothermia, and fever were compared, and fold-change was calculated if statistically significant changes in ΔCt existed.

Fold-change was calculated as $2^{(S_{avg}\Delta Ct - C_{avg}\Delta Ct)}$, where $S_{avg}\Delta Ct - C_{avg}\Delta Ct$ was the difference between the sample (heated ΔCt) and control (non-heated ΔCt) at a specific time point.
**Indomethacin treatment.** To determine the role of COX pathway activation on the delayed fever response during HS recovery, mice were treated orally with indomethacin (5 mg/kg contained within a bacon-flavored oral treat; Bio-Serv, Frenchtown, NJ) and the fever response compared to mice that received a bacon-flavored treat containing no drug. Mice were heated to a lower Tc,Max (42.4°C) than used in the gene expression study to ensure survival and fever development the day following HS, as previously described (20). Treats were placed onto the cage floor ~24h following the start of heat exposure when fever was observed. Indomethacin is a non-selective inhibitor of COX-1 and -2 enzymes that participate in prostaglandin (PG) synthesis for the development of fever during peripheral inflammation in mice (17). The dose of indomethacin used in this experiment was shown previously to abolish fever to a high dose of LPS in mice, which is mediated by IL-1β, IL-6, TNFα and COX activation in this species (17, 18). We chose an oral route of indomethacin administration to minimize stress effects associated with handling and injection (e.g., hyperthermia), which were expected to confound analysis of the fever response (2). Oral consumption of the treat was verified for each mouse and occurred within ~5 min of placement onto the cage bottom.

**Statistical Analysis:** Gene expression data are presented as mean fold-change with SE. At each time point, unpaired t-tests or Mann-Whitney Rank Sum tests were conducted on normalized Ct values to compare HS to non-heated time point control animals. To compare gene expression changes in HS animals across time, Kruskal-Wallis One Way Analysis of Variance on Ranks with Dunns’ Method for pairwise comparisons were used. All statistics were performed with Sigma Plot 12.0 (Systat Software, San Jose, CA), and P < 0.05 was considered significant.
Fever and activity data were analyzed using Two-Way Analysis of Variance (ANOVA) followed by the Holm-Sidak method for multiple comparisons.

**Results:**

*Tc and activity profile of HS mice:* Figure 1A shows the ~48-h Tc profile of a representative non-heated control (bold line) and HS (thin line) mouse. Details of the heating profile have been provided elsewhere (19). Briefly, mice remained in the heat stress environment for ~252 min until Tc,Max of 42.7°C was reached. HD and fever were observed at ~115 min and ~1200 min, respectively, following removal from the heat at Tc,Max. Mice were sacrificed at baseline, Tc,Max, HD or fever for brain harvesting and gene expression analysis (sampling time points indicated on graph).

Figure 1B shows the home-cage activity profile of non-heated control (bold line) and HS mice (thin line) through ~48-h of HS recovery. Non-heated control mice displayed a circadian activity profile with low daytime (lights-on, inactive period) and high nighttime (lights-off, active period) levels representative of this species. Activity was significantly higher in HS mice during the initial ~3h of heat exposure as mice showed considerable movement within the cage during this period that was indicative of escape behavior (1-3h; Figure 1B; ANOVA, P<0.001). During cooling to HD, activity levels were significantly reduced in HS mice and this response was maintained throughout the first lights-off period (9h-15h, Figure 1B; ANOVA, P<0.05). Activity levels during the next lights-on period were similar between groups. HS mice continued to show significantly attenuated activity levels compared to non-heated controls during the second lights-off period, even though Tc was similar between groups during this time period (33-38h, Figure 1A and B; ANOVA, P<0.05; (20).
HSP gene expression changes: Non-heated control mice maintained similar levels of expression in the hypothalamus and hippocampus for all genes examined throughout HS recovery (data not shown). HS mice showed a progressive increase in HSP72 gene expression in the hypothalamus from \( T_{c,\text{Max}} \) (43.6 ± 11.5 fold-change; Student’s test, \( P=0.003 \)) through HD (216.5 ± 18 fold-change; Student’s test, \( P=0.01 \)), with levels significantly higher than non-heated controls at both time points of recovery (Figure 2A). HSP72 gene expression no longer differed between non-heated control and HS mice at the time of fever (Figure 2A).

HSP72 gene expression in the hippocampus of HS mice was significantly higher than non-heated controls from \( T_{c,\text{Max}} \) (174.7 ± 34 fold-change) through HD (668.9 ± 16 fold-change; Figure 2B; Student’s t-test, \( P<0.05 \)). During fever, HSP72 gene expression of HS mice was virtually identical to non-heated controls in this brain region. There was a main effect of time on HSP72 with HD levels significantly higher than \( T_{c,\text{Max}} \) and fever (\( P<0.05 \)). The hippocampus of HS mice showed a more robust increase in HSP72 gene expression at \( T_{c,\text{Max}} \) and HD than that observed in the hypothalamus (Figure 2A and B; ANOVA, \( P<0.001 \)).

As observed with HSP72, hmox1 gene expression was also differentially increased in the hypothalamus and hippocampus. HS mice showed a significant increase of hmox1 at HD only in both regions, but this response was more robust in the hippocampus than the hypothalamus (11.4 ± 1.3 fold-change vs. 7.2 ± 0.4 fold-change, respectively; Figure 2C and D; ANOVA, \( P<0.001 \)).

Cytokine gene expression changes: IL-1\( \beta \) gene expression in the hypothalamus of HS mice was increased above non-heated controls from \( T_{c,\text{Max}} \) (3.7 ± 0.8 fold-change; \( P=0.038 \)) to HD (26.8 ± 5.3 fold-change; \( P=0.006 \); Figure 3A). Conversely, IL-6 (3.6 ± 1.1 fold-change;
P=0.01) and TNF-α (7.8 ± 1.9 fold-change; P=0.024) gene expression did not increase until HD (Figure 3A). During fever, all cytokine gene expression changes of HS mice in the hypothalamus were similar to non-heated controls. The increase in IL-1β and IL-6 at Tc,Max and HD were similar to one another, but significantly elevated above the response observed at the time of fever (P<0.05). The highest level of TNF-α expression was observed at HD, which represented a significant difference from Tc,Max, but not fever (P<0.05).

Unlike the hypothalamus, IL-1β gene expression did not increase in the hippocampus of HS mice until HD (12.0 ± 2.1 fold-change, Figure 3B; Student’s t-test, P<0.05). IL-6 and TNFα gene expression was also increased at this time point (11.8 ± 5.2 and 6.4 ± 1.3 fold-change, respectively; P<0.05). During fever, IL-1β, IL-6 and TNFα gene expression was similar to non-heated controls in the hippocampus (Figure 3B). There was a significant effect of time on IL-1β, IL-6, and TNF-α gene expression (all P<0.001) with IL-β and TNFα levels at HD greater than Tc,Max (P<0.05), but not fever. IL-6 gene expression at HD was higher than the level observed at Tc,Max and fever (P<0.05). The only regional difference in HS mice was observed in IL-1β with higher gene expression levels observed at HD in the hypothalamus than the hippocampus (Figure 3A and B; ANOVA, P=0.017).

**COX gene expression changes:** COX-1 gene expression was virtually identical between non-heated control and HS mice at all time points of recovery in both brain regions (data not shown). The only increase in COX-2 gene expression in the hypothalamus of HS mice occurred at HD (2.8 ± 0.5 fold-change, Figure 3C; Student’s t-test, P=0.01). In the hippocampus, COX-2 appeared to show a robust increase at Tc,Max, but this response was highly variable and did not represent a significant response compared to non-heated controls at this time point (Figure 3D;
Student’s t-test, P=0.16). As such, COX-2 was not increased in the hippocampus of HS mice at any time point of recovery. There were no regional differences in the COX-2 response to HS.

Chemokine and chemokine receptor gene expression changes: MCP-1, MIP-1α and MIP-1β gene expression was similar in the hypothalamus of non-heated control and HS mice at $T_{c,\text{Max}}$; however, HS mice showed a significant increase in gene expression of all of these chemokines compared to non-heated controls at HD ($7.2 \pm 1.7$, $23.7 \pm 2.2$, and $42.9 \pm 4.6$ fold-change, respectively; Figure 3E; Student’s t-test, $P<0.05$). There was a significant effect of time on MCP-1, MIP-1α, and MIP-1β with all genes showing upregulation at HD compared to $T_{c,\text{Max}}$ and fever (ANOVA, $P<0.05$). Conversely, CX3CR1 gene expression was significantly decreased from $T_{c,\text{Max}}$ (-4.7 fold-change; ANOVA, $P=0.01$) through HD (-5.8 fold-change; ANOVA, $P<0.001$) with a return to baseline levels at the time of fever (Figure 3E).

Similar to the changes observed in the hypothalamus, MCP-1, MIP-1α and MIP-1β were increased above non-heated controls in the hippocampus of HS mice at HD only ($15.7 \pm 4.8$, $11.0 \pm 4.2$, and $25.0 \pm 5.6$ fold-change, respectively; Figure 3F; Student’s t-test, $P<0.05$). MCP-1 levels at HD were higher than $T_{c,\text{Max}}$ (ANOVA, $P<0.05$), but not fever. On the other hand, MIP-1α and -β gene expression at HD was higher than all other time points (ANOVA, $P<0.05$). CX3CR1 gene expression was significantly decreased in HS compared to non-heated control mice at $T_{c,\text{Max}}$ (-4.0 fold-change; $P<0.01$) and HD (3.0 fold-change; $P<0.01$), but returned to control levels at the time of fever (Figure 3F). There were no regional differences in the chemokine or CX3CR1 response to HS at any time point of recovery.
Glia activation marker gene expression changes: CD14 gene expression in the hypothalamus was unaffected by heat at $T_{c,\text{Max}}$, but showed significant upregulation at HD (8.6 ± 2.8 fold-change) and a return to baseline by the time of fever (Figure 4A; Student’s t-test, $P<0.001$). TLR4 was virtually identical to non-heated controls at all time points of recovery in the hypothalamus (data not shown). Aif1 gene expression in the hypothalamus of HS mice was downregulated compared to non-heated controls at $T_{c,\text{Max}}$ (-2.6 ± 0.5 fold-change; $P<0.001$) and HD (-1.9 ± 0.2 fold-change; $P=0.003$) with a return to baseline levels at the time of fever (Figure 4A; $P<0.05$). Vimentin gene expression was virtually identical between non-heated control and HS mice at all time points of recovery in this brain region (Figure 4A).

In the hippocampus, CD14 was elevated at HD only (4.7 ± 1.1 fold-change; Student’s t-test, $P<0.01$) and returned to non-heated control levels by the time of fever (Figure 4B). TLR4 was virtually identical to non-heated controls at all time points of recovery in the hippocampus (data not shown). Aif1 showed no gene expression changes during HS recovery whereas vimentin was increased at HD only (3.2 ± 0.6 fold-change; Figure 4B; Student’s t-test, $P<0.01$). There were no regional differences in the glia activation marker responses to HS at any time point of recovery.

Effect of indomethacin on fever during HS recovery. The $T_c$ profile displayed by mice during the ~24-48h period of HS recovery is depicted in Figure 5A. Non-heated control mice orally treated with a bacon treat containing no drug or 5 mg/kg indomethacin showed virtually identical circadian $T_c$ profiles so these data were combined into one group for ease of presentation. Non-heated controls (bold line) showed the normal circadian $T_c$ profile with low daytime (lights-on period; $\sim36^\circ\text{C}$) and high nighttime values (lights-off period; $\sim37^\circ\text{C}$; Figure
The ~1°C transient increase in $T_c$ at ~24h represents stress-induced hyperthermia in response to disturbance during placement of the oral treat onto the cage bottom, which was observed in all groups. HS mice orally treated with a bacon treat containing no drug showed the typical fever response during the lights-on period, which was a sustained elevation of $T_c$ above non-heated controls (24-36h; 36.7 ± 0.2 vs. 36.1 ± 0.1°C, respectively; ANOVA, P<0.05) from ~24-36h; oral treatment with indomethacin had no effect on the fever response (37.0 ± 0.1°C; Figure 5A). The daytime (lights-on; 12-h ave.) $T_c$ response of non-heated control, no drug- and indomethacin-treated mice is shown in Figure 5B. Home-cage activity was significantly decreased in HS mice during the lights-off period with no effect of indomethacin on this response (data not shown).

**Discussion:**

This is the first study to identify the time course of changes in gene expression of HSPs, cytokines, COX enzymes, chemokines/chemokine receptors and glia activation markers in the hypothalamus and hippocampus of conscious mice during 24-h of HS recovery. Both temporal and regional differences in gene expression changes were observed with the most robust responses occurring at ~2h of HS recovery when mice displayed hypothermia. Despite several clinical reports suggesting that hypothermia is a consequence of heat-induced damage to the POAH, we showed previously that neither the POAH nor hippocampus was damaged in our mouse model at HD (21). While our data do not provide direct evidence for a cause and effect role of the inflammatory mediators measured in this study in regulation of the $T_c$ changes or sickness behaviors during HS recovery, there was a close temporal association between hypothermia and the most robust gene expression changes. Importantly, the time course of gene
expression changes observed throughout recovery were not identical between the hypothalamus and hippocampus, suggesting these were not simply global CNS responses that were unrelated to the underlying T_c changes or sickness behaviors. Perhaps most interesting was the observation that the most robust gene expression changes were observed at HD, which preceded the development of fever in our model. As such, some of these changes may represent the thermoregulatory “signal” that regulated HD or initiated recovery for development of the delayed fever response. In mice, the peripheral injection of LPS induces a biphasic T_c response consisting of hypothermia and delayed fever that is remarkably similar to that observed during HS recovery (18). IL-6, TNFα and PGs have been implicated in the regulation of LPS-induced hypothermia and fever in mice, but remains unknown if similar mechanisms are operative during HS recovery (17).

Bidirectional communication between the peripheral immune system and brain mediates sickness behaviors (e.g., fever, lethargy) during recovery from infection and/or inflammation. These responses are thought to be a consequence of peripheral changes in cytokines, such as IL-1β, IL-6 and TNFα that induce central cytokine, chemokine and PG production, as well as glia activation (11). Following HS we observed a long-term suppression of home-cage activity and increases in CNS cytokines and chemokines that are consistent with a role for central cytokines/chemokines in mediating the behavioral responses to HS. Future studies should examine changes in cytokine and chemokine proteins in the hypothalamus following HS. Ultimately, it would be most informative to conduct targeted studies using pharmacological or genetic disruption of cytokine and chemokine signaling in the hypothalamus to demonstrate the causal role of these factors in mediating the physiological and behavioral consequences of HS.
CNS damage has been implicated as a causal factor for the development of hypothermia and/or delayed fever during HS recovery in patients and animal models despite scant evidence to support this hypothesis (21, 25). We observed a significant increase in HSP72 and the microglia/macrophage activation marker CD14 in the hypothalamus and hippocampus of mice at HD despite an inability previously to detect histological damage to these CNS regions at this time point in our model (21). Reductions in the expression of Aif1 and CX3CR1 were also observed which suggests glia cells and/or CNS infiltrating macrophages were affected by HS but the functional implications of these gene expression reductions remain unknown. Given the lack of histological damage in our model, the gene expression changes we observed were likely not a pathophysiological response to damage per se, but represented regulated changes that may have affected the temperature balance point (30).

Glia cells are a major source of CNS cytokines and chemokines and could play a major role in the thermoregulatory changes following HS. One of the interesting aspects of the Tc response to HS in mice that has eluded explanation is the ability of these animals to recover from hypothermia and develop fever following a heat insult. What is the trigger for hypothermia rewarming and fever development during HS recovery? Based on the evidence provided in this study, we hypothesize that increased cytokine, chemokine and/or PG gene expression in the hypothalamus may be the physiological mechanism that induces hypothermia and/or an increase in metabolic rate for the development of fever in our model. We showed previously that recovery from HD and development of fever during HS recovery was associated with ~20% increase in metabolic rate, but the mechanism(s) stimulating this response was not identified (21). Several cytokines (IL-1β, IL-6) and chemokines (MIP-1α, MIP-1β) act as endogenous pyrogens in the hypothalamus during bacterial infection. For example, direct injection of IL-1β
into the brain increases body temperature and non-REM sleep (28). Furthermore, IL-1 mRNA and protein are synthesized in the hypothalamus in response to peripheral pyrogenic stimuli such as LPS (15, 24) as well as HS (22). Perhaps increased cytokine or chemokine gene expression at HD is the initiating event for re-warming from HD during HS recovery in mice.

We previously demonstrated that many cytokine knockout mice (e.g., IL-1RI, IL-6 and TNF receptor) develop fever during HS recovery, which suggests that either developmental redundancy occurred in these mice or substances other than cytokines were mediating the delayed fever response (14). IL-6 and COX-2 have been implicated in the regulation of fever in mice based on studies showing an inhibitory effect of indomethacin on this response (17). To determine the role of the COX-2 pathway in the mediation of fever during HS recovery, we examined the effect of oral indomethacin on this response. The lack of an effect of indomethacin on fever is congruent with our gene expression data that failed to show an increase in COX-2 or the cytokines known to induce this enzyme (e.g., IL-6) at this time point. As such, COX-2 and downstream PG production in the hypothalamus does not appear to be mediating fever during HS recovery. Taken together with the observation of increased chemokine expression at HD, we hypothesize that the delayed fever during HS recovery is a PG-independent event that may be stimulated by chemokines or other unidentified pyrogens within the hypothalamus. Indeed, injection of MIP-1α or MIP-1β into the hypothalamus can induce febrile responses in a PG-independent manner (12, 27, 32) and is consistent with our hypothesis. While historically thought of as leukocyte recruitment signals, the demonstration of constitutive expression of chemokines and chemokine receptors on a variety of CNS cell types (microglia, astrocytes, oligodendrocytes, neurons and brain microvasculature) suggests they may serve as potent modulators of neural function during homeostasis, homeostatic challenge, and
neuroinflammation (8). Several of the neuroendocrine functions altered by HS, such as $T_c$
regulation, water balance and feeding behavior have been found to be sensitive to manipulations
of chemokine signaling (7). These findings, along with our demonstration of robust chemokine
changes during hypothermia, suggest additional examination of the function of CNS chemokines
in HS recovery is warranted and may provide novel therapeutic targets for investigation.

Of particular relevance to the long-term health outcomes associated with recovery from
HS is recent evidence that acute increases in brain cytokines can set in motion a prolonged
sensitization of glia cells (3, 10, 13, 29). This sensitization, known as glia priming, can lead to a
potentiated pro-inflammatory response upon subsequent secondary insults (i.e. peripheral
immune challenge, stress, injury, etc.) and may play a role in the development of
neurodegenerative disease. The changes in CD14, CX3CR1 and vimentin mRNA are suggestive
of glia alterations following HS but future immunohistochemical analysis is necessary to
elucidate the cell types involved.

In summary, we report significantly increased expression of pro-inflammatory cytokines,
chemokines and glia activation markers in the hypothalamus and hippocampus of mice that are
associated with $T_c$ changes and lethargy during HS recovery. These changes predominately
occur at HD, suggesting a potential role in mediating this response and the subsequent elevation
in temperature balance point and fever development that occurs during HS recovery. In
agreement with such an interpretation is the observation that an inability to recover from
hypothermia is associated with enhanced mortality and the changes in gene expression observed
here all suggest an attempt to elevate $T_c$. Of importance to understanding the long-term
repercussions of recovery from HS, the acute increase in chemokines and cytokines may also
sensitize the brain to future insult and therefore could have important implications for the CNS response to later stressors.
Acknowledgements

We thank M. Condlin for technical assistance with tissue preparation and RT-PCR.

Research was funded by U.S. Army Medical Research and Materiel Command. This article has been approved for public release: distribution is unlimited.

No conflicts of interest, financial or otherwise, are declared by the authors. The opinions or assertions contained herein are the private views of the author(s) and are not to be construed as official or reflecting the views of the Army or the Department of Defense.

Any citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement of approval of the products or services of these organizations.
Figure 1: (A) Representative core temperature profile of a non-heated control (bold line) and heat stroke (HS; thin line) C57BL/6J mouse through 48h of experimentation. HS is associated with hyperthermia (from baseline to $T_c$,Max) followed by a biphasic thermoregulatory response consisting of cooling to hypothermia depth (HD) and rewarming for the development of delayed fever from ~24-36h. Mice were sacrificed at baseline (immediately prior to heat exposure), $T_c$,Max, HD and fever (~24h after the start of heat exposure) for brain harvesting to support gene expression analysis. (B) Home-cage activity of C57BL/6J non-heated control (bold line) or HS mice (thin line) during 48h of HS recovery. HS mice showed a significant suppression of nighttime (lights-off; black horizontal bars) activity compared to non-heated controls. *Significant difference in activity counts of HS mice compared to non-heat controls at P<0.05. $T_c$,Max, final $T_c$ during heating; HD, calculated as lowest 1h average $T_c$ observed during recovery. Black bar represents lights-off (active) period.

Figure 2: Changes in hypothalamus (left side) and hippocampus (right side) gene expression of hsp-72, (A,B) and hmxo1 (C,D) at $T_c$,Max, hypothermia depth (HD), and the time of fever during HS recovery. Gene expression was measured by real-time PCR. Graphs represent mean fold-change relative to non-heated, time-point control. Range of fold-change values are provided below each figure. a = HS significantly different from unheated control; b = HD significantly different from $T_c$,Max; c = $T_c$,Max significantly different from fever; d = HD significantly different from fever. Significance set at P<0.05.

Figure 3: Changes in hypothalamus (left side) and hippocampus (right side) gene expression of IL-1β, IL-6, TNFα (A,B), COX-2 (C,D), MCP-1, MIP-1α, MIP-1β, CX3CR1 (E,F) at $T_c$,Max, hypothermia depth (HD), and the time of fever during HS recovery. Gene expression was measured by real-time PCR. Graphs represent mean fold-change relative to unheated, time-point control. Range of fold-change values are provided below each figure. a = HS significantly different from unheated control; b = HD significantly different from $T_c$,Max; c = $T_c$,Max significantly different from fever; d = HD significantly different from fever. Significance set at P<0.05.

Figure 4. Changes in hypothalamus (A) and hippocampus (B) gene expression of the glia activation markers CD14, Aif1, and vimentin at $T_c$,Max, hypothermia depth (HD), and the time of fever during HS recovery. Gene expression was measured by real-time PCR. Graphs represent mean fold-change relative to unheated, time-point control. Range of fold-change values are provided below each figure. a = HS significantly different from unheated control; b = HD significantly different from $T_c$,Max; c = $T_c$,Max significantly different from fever; d = HD significantly different from fever. Significance set at P<0.05.

Figure 5. Core temperature circadian profile (A) and daytime (light-on, 12h-ave) $T_c$ response (B) of non-heated control and HS mice treated with no drug or indomethacin (5 mg/kg) at the time of fever (~24h after the start of heat exposure). Non-heated control mice orally treated with no drug or indomethacin showed virtually identical $T_c$ responses so these data were combined into one group for ease of presentation. Indomethacin had no effect on the ~24-36h fever response during HS recovery. *represents significant increase in $T_c$ compared to non-heated controls at P<0.05. Black bar represents lights-off (active) period.
References:


<table>
<thead>
<tr>
<th>Gene nomenclature:</th>
<th>Common Name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Heat shock proteins:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSP72</td>
<td>Heat shock protein 72</td>
<td>Marker of thermal stress; molecular chaperone of denatured proteins</td>
</tr>
<tr>
<td>Hmox1</td>
<td>Heme oxygenase 1</td>
<td>Marker of cellular oxidative stress</td>
</tr>
<tr>
<td><strong>Cytokines:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1β</td>
<td>Endogenous pyrogen</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
<td>Endogenous pyrogen</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor α</td>
<td>Endogenous antipyretic</td>
</tr>
</tbody>
</table>

*These cytokines stimulate or attenuate fever via interactions with the cyclooxygenase 2 pathway*

| **Cyclooxygenase:** | | |
| COX-1 | Cyclooxygenase-2 | Constitutive COX enzyme |
| Ptgs2 (COX-2) | Prostaglandin endoperoxide synthase 2, also known as COX-2 | Inducible COX enzyme |

| **Chemokines and receptors:** | | |
| CCL2 | Monocyte chemoattractant protein (MCP)-1 | Chemoattractant (monocytes) |
| CCL3 | Macrophage inflammatory protein (MIP)-1α | Endogenous pyrogen |
| CCL4 | Macrophage inflammatory protein (MIP)-1β | Endogenous pyrogen |
| CX3CR1 | Fractalkine receptor | Chemoattractant (microglia) |

*MIP-1α and β induce fever independently of COX activation*

| **Glial Activation Markers:** | | |
| CD14 | Lipopolysaccharide receptor | CD14 and TLR4 are co-receptors that participate in lipopolysaccharide detection |
| TLR4 | Toll-like receptor 4 | |
| Aif1 | Allograft inflammatory factor-1 | Marker of reactive microglia |
| Vimentin | Intermediate filament protein | Marker of reactive astrocytes |
Figure 1

A

Core Temperature (°C)

- Tc,Max
- Fever
- Baseline
- HD
- Non-heated Control
- Heat Stroke

0 12 24 36 48

Time After Start of Heat Exposure

B

Activity (Counts)

- Tc,Max
- Fever
- Baseline
- HD
- *

0 12 24 36 48

Time After Start of Heat Exposure
Figure 3

**Hypothalamus**

<table>
<thead>
<tr>
<th></th>
<th>Tc,Max</th>
<th>HD</th>
<th>Fever</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>1.2-5.8</td>
<td>10.4-48.0</td>
<td>0.9-5.8</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.5-2.6</td>
<td>1.7-7.8</td>
<td>0.2-13</td>
</tr>
<tr>
<td>TNFα</td>
<td>0.2-6.6</td>
<td>3.0-15.7</td>
<td>1.1-4.8</td>
</tr>
</tbody>
</table>

**Hippocampus**

<table>
<thead>
<tr>
<th></th>
<th>Tc,Max</th>
<th>HD</th>
<th>Fever</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>0.2-1.5</td>
<td>3.9-18.7</td>
<td>0.8-19</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.1-3.8</td>
<td>1.7-10.6</td>
<td>0.4-4.5</td>
</tr>
<tr>
<td>TNFα</td>
<td>0.2-1.9</td>
<td>2.3-10.3</td>
<td>0.6-17.2</td>
</tr>
</tbody>
</table>

**Cox-2**

<table>
<thead>
<tr>
<th></th>
<th>Tc,Max</th>
<th>HD</th>
<th>Fever</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8-4.4</td>
<td>2.8-5.6</td>
<td>0.7-4.5</td>
<td></td>
</tr>
</tbody>
</table>

**MCP-1**

<table>
<thead>
<tr>
<th></th>
<th>Tc,Max</th>
<th>HD</th>
<th>Fever</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4-2.4</td>
<td>3.0-13.6</td>
<td>0.4-4.5</td>
<td></td>
</tr>
</tbody>
</table>

**MIP-1α**

<table>
<thead>
<tr>
<th></th>
<th>Tc,Max</th>
<th>HD</th>
<th>Fever</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8-2.0</td>
<td>15.1-50.1</td>
<td>0.1-1.5</td>
<td></td>
</tr>
</tbody>
</table>

**MIP-1β**

<table>
<thead>
<tr>
<th></th>
<th>Tc,Max</th>
<th>HD</th>
<th>Fever</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9-4.3</td>
<td>23.5-55.3</td>
<td>0.8-2.7</td>
<td></td>
</tr>
</tbody>
</table>

**CX3CR1**

<table>
<thead>
<tr>
<th></th>
<th>Tc,Max</th>
<th>HD</th>
<th>Fever</th>
</tr>
</thead>
<tbody>
<tr>
<td>-3.4 to -6.8</td>
<td>-3.2 to -14.2</td>
<td>0.8 to 1.8</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Tc,Max</th>
<th>HD</th>
<th>Fever</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5-2.6</td>
<td>7.6-42.5</td>
<td>0.6-17.2</td>
<td></td>
</tr>
<tr>
<td>0.9-2.5</td>
<td>3.1-3.05</td>
<td>0.5-3.7</td>
<td></td>
</tr>
<tr>
<td>1.8-4.5</td>
<td>9.4-60.1</td>
<td>0.9-3.7</td>
<td></td>
</tr>
<tr>
<td>-1.8 to -6.8</td>
<td>-1.6 to -6.6</td>
<td>0.3-1.7</td>
<td></td>
</tr>
</tbody>
</table>
**Hypothalamus**

**Hippocampus**

**Figure 4**

![Graph A](image)

![Graph B](image)

<table>
<thead>
<tr>
<th>Protein</th>
<th>CD14 Range</th>
<th>AIF1 Range</th>
<th>VIMENTIN Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tc,Max</td>
<td>0.6-3.7</td>
<td>3.6-21.9</td>
<td>1.1-2.2</td>
</tr>
<tr>
<td>HD</td>
<td>-1.3-(-5.3)</td>
<td>(-1.3)-(-2.4)</td>
<td>(-9.2-2.1)</td>
</tr>
<tr>
<td>Fever</td>
<td>0.5-1.2</td>
<td>0.6-3.4</td>
<td>1.1-2.7</td>
</tr>
</tbody>
</table>

**Range of Fold-Change**

- CD14: 0.7-1.4
- AIF1: 0.6-1.4
- VIMENTIN: 0.4-1.3
Figure 5

A

Core Temperature (°C)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Group Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-heated Controls (N=18)</td>
<td></td>
</tr>
<tr>
<td>No Drug Heat (N=9)</td>
<td></td>
</tr>
<tr>
<td>125 µg Indomethacin (N=9)</td>
<td></td>
</tr>
</tbody>
</table>

Oral tx

LIGTHS OFF

24h           36h

Time After Heat Exposure (1 Min Aves)

B

Core Temperature (°C)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Core Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-heated Control</td>
<td>36.0</td>
</tr>
<tr>
<td>No Drug Heat</td>
<td>*</td>
</tr>
<tr>
<td>Indomethacin Heat</td>
<td>*</td>
</tr>
</tbody>
</table>