HIGHLIGHTED TOPIC | A Physiological Systems Approach to Human and Mammalian Thermoregulation

Time course of cytokine, corticosterone, and tissue injury responses in mice during heat strain recovery

Lisa R. Leon, Michael D. Blaha, and David A. DuBose

Thermal and Mountain Medicine Division, United States Army Research Institute of Environmental Medicine, Natick, Massachusetts

Submitted 25 August 2005; accepted in final form 15 October 2005

Leon, Lisa R., Michael D. Blaha, and David A. DuBose. Time course of cytokine, corticosterone, and tissue injury responses in mice during heat strain recovery. J Appl Physiol 100: 1400–1409, 2006. First published October 20, 2005; doi:10.1152/japplphysiol.01040.2005.—Elevated circulating cytokines are observed in heatstroke patients, suggesting a role for these substances in the pathophysiological responses of this syndrome. Typically, cytokines are determined at end-stage heatstroke such that changes throughout progression of the syndrome are poorly understood. We hypothesized that the cytokine milieu changes during heatstroke progression, correlating with thermoregulatory, hemodynamic, and tissue injury responses to heat exposure in the mouse. We determined plasma IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p40, IL-12p70, IFN-γ, macrophage inflammatory protein-1α, TNF-α, corticosterone, glucose, hematocrit, and tissue injury during 24 h of recovery. Mice were exposed to ambient temperature of 39.5 ± 0.2°C, without food and water, until maximum core temperature (Tc,Max) of 42.7°C was attained. During recovery, mice displayed hyperthermia (29.3 ± 0.4°C) and a feverlike elevation at 24 h (control = 56.2 ± 0.3°C vs. heat stressed = 37.8 ± 0.3°C). Dehydration (~10%) and hypoglycemia (~65–75% reduction) occurred from Tc,Max to hypothermia. IL-1α, IL-2, IL-4, IL-12p70, IFN-γ, TNF-α, and macrophage inflammatory protein-1α were undetectable. IL-12p40 was elevated at Tc,Max, whereas IL-1β, IL-6, and IL-10 inversely correlated with core temperature, showing maximum production at hypothermia. IL-6 was elevated, whereas IL-12p40 levels were decreased below baseline at 24 h. Corticosterone positively correlated with IL-6, increasing from Tc,Max to hypothermia, with recovery to baseline by 24 h. Tissue lesions were observed in duodenum, spleen, and kidney at Tc,Max, hypothermia, and 24 h, respectively. These data suggest that the cytokine milieu changes during heat strain recovery with similarities between findings in mice and those described for human heatstroke, supporting the application of our model to the study of cytokine responses in vivo.

Heatstroke; hypothermia; fever; systemic inflammatory response

Heatstroke is a life-threatening illness clinically defined as core temperature (Tc) > 41.0°C, hot, flushed dry skin, and the presence of central nervous system abnormalities, such as delirium, convulsions, and coma (5). Despite clinical cooling therapies to minimize tissue injury, permanent neurological damage is incurred in up to ~30% of heatstroke survivors (10). A more thorough understanding of the physiological mechanisms mediating thermal injury is needed for the development of clinical strategies to minimize the adverse consequences of this syndrome.

Current data suggest that the pathophysiological responses to heatstroke may not be due to the immediate effects of heat exposure, per se but the result of a systemic inflammatory response syndrome (SIRS) that ensues following thermal injury (5). Cytokines are important regulators of the acute phase response to inflammation/injury and have been implicated as mediators of SIRS with heatstroke. There are several lines of evidence supporting a role for cytokines in heat-induced SIRS, including 1) increased circulating levels of cytokines in patients and experimental animals at end-stage heatstroke (2–4, 6, 21), 2) beneficial effects of IL-1 antagonism on morbidity and mortality of heatstroke in rats (the effectiveness of antagonism of other cytokines has not been reported, and cytokine neutralization has not been examined in other species; Ref. 22), and 3) induction of heat shock symptoms (e.g., hypotensive shock and tissue injury) following IL-1 or TNF injection (22, 32). Indirect evidence for a role of endogenous cytokines in heatstroke includes the association of endotoxemia with heatstroke and the known role of cytokines in the endotoxemic syndrome (2, 12, 14, 16–18, 29, 31).

At the time of clinical admission, elevations in circulating concentrations of IL-1α, IL-1β, IL-1 receptor antagonist, IL-6, IL-10, IFN-γ, and TNF-α are observed in heatstroke patients (2, 3, 4, 9). In some cases, only 30–40% of patients show increased concentration of a particular cytokine (e.g., IL-1β and IL-10; Refs. 3, 4), whereas other cytokines, such as IL-6, are often significantly elevated in 100% of patients (3). Variability in cytokine responses may be indicative of differential roles of these mediators in the heatstroke syndrome or may be a consequence of the wide variability in time of clinical presentation among patients. The observation that pro- (e.g., IL-1) and anti-inflammatory (e.g., IL-10) cytokines are elevated concomitantly suggests a complex network of interactions in the manifestation of heat-induced SIRS. Unfortunately, due to circulating cytokines being determined primarily at end-stage heatstroke, knowledge regarding the time course of changes in the balance of these mediators during progression of the syndrome is limited. A determination of cytokine expression patterns over time is needed to provide insight into specific cytokines to target using neutralization techniques and
to support a mechanistic approach to understanding the role of these mediators in the heat syndrome.

Several attempts have been made to correlate cytokine changes with different aspects of the heat syndrome, such as the degree of hyperthermia. It is typically difficult to find a strong correlation between cytokine levels and Te in human heatstroke cases, because of variable presentation times and clinical cooling interventions (2–4). Interestingly, in one study, the ability to cool patients from 40 to 38°C was dependent on serum IL-1β levels (9). This is the only report directly implicating endogenous IL-1β or any cytokine in the control of Te responses in heatstroke patients. This is surprising since several of the cytokines implicated in heatstroke pathophysiology are known regulators of Te in health and disease (16, 18).

The aim of this study was to examine changes in the time course of cytokine production during heat strain recovery in mice and correlate these changes with thermoregulatory, hemodynamic, and tissue injury responses. Using a previously established mouse model of severe heat strain (20), we measured the plasma level of 11 cytokines, some of which have been previously implicated in heatstroke pathophysiology (IL-1α, IL-β, IL-2, IL-6, IL-10, IFN-γ, TNF-α), as well as additional cytokines known to have a role in SIRS with infection and inflammatory tissue injury [IL-4, IL-12p40, IL-12p70, macrophage inflammatory protein (MIP)-1α]. Additional measures included plasma corticosterone, due to its endogenous function as a natural inhibitor of cytokine production, and tissue injury histopathology, as it reflects a common manifestation of SIRS in heatstroke.

The choice of a mouse model for these studies was based on the following considerations. 1) We recently developed a mouse model of heat strain that permits the determination of cytokine levels in conscious, free-ranging animals, thus being more representative of natural heatstroke conditions (most studies use anesthetized or otherwise physiologically or behaviorally compromised animal models; Refs. 6, 21, 22). 2) Mice develop a biphasic thermoregulatory response during heat strain recovery that consists of initial hyperthermia and a subsequent febrile Te elevation (20); the ability to precisely target these Te phases in conscious animals allowed us to determine the correlation between cytokine production and Te during heat strain progression and recovery. 3) The identification of cytokine changes in a mouse model supports future studies to examine the role of individual cytokines in heatstroke pathophysiological responses using neutralizing antibodies or gene knockout models.

MATERIALS AND METHODS

Animals. Specific pathogen-free male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME), weighing 30.8 ± 0.2 g (2–3 mo old) on the day of experimentation, were used. Mice were individually housed in Nalgene polycarbonate cages (11.5 × 7.5 × 5 in.), fitted with HEPA filter cage tops and wood chip bedding (Pro-Chip, PWI). Rodent laboratory chow (Harlan Teklad, LM-485, Madison, WI) and water were provided ad libitum under standard laboratory conditions (25 ± 2°C; 12:12-h light-dark cycle, lights on at 0700). 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.

$T_c, T_e$ (±0.1°C) was continuously monitored at 1-min intervals by biotelemetry in conscious, freely moving mice using the Dataquest A.R.T. system (Data Sciences International, St. Paul, MN). Briefly, for transmitter implantation, each animal was anesthetized with isoflurane (4% induction, 2.5% maintenance in 100% O2, flow rate = 0.7 l/min), and a temperature-sensitive transmitter (model TA10TA-F20) was implanted intra-abdominally using aseptic technique. Frequency of the emitted transmitter signal is proportional to $T_e$. An antenna placed under each animal’s cage received the emitted transmitter signal and converted it to $T_e$ using predetermined calibration values. Each transmitter was magnetically activated ≥1 wk before experimentation to ensure a stable circadian $T_e$ rhythm before heat exposure. Transmitters were calibrated before and after experimentation to ensure accuracy of $T_e$ measurements.

Transmitter weights were ~3.6 g, which represented ~12% of mouse body weight (BW). Ibuprofen analgesia (Children’s Advil, Cold Formula, grape, Wyeth Healthcare, El Paso, TX) was provided in the drinking water (200 μg/ml) 24 h before surgery and by injection immediately following transmitter implantation (50 mg/kg sc). Surgical recovery (~14 days) was required before heat stress experimentation, as defined by a return to presurgical BW and manifestation of a robust, consistent circadian $T_e$ rhythm, as previously described for this species (19).

Heat stress and sampling protocol. The heat stress protocol has been described in detail elsewhere (20). Briefly, conscious, unrestrained mice were exposed to an ambient temperature ($T_a$) of 39.5 ± 0.2°C in an incubator, in the absence of food and water, until a maximum $T_e$ ($T_{e,\text{Max}}$) of 42.7°C was attained. Following removal from the heat at $T_{e,\text{Max}}$, food and water were provided ad libitum during undisturbed recovery at $T_a$ of 25 ± 2°C. This relatively cool recovery $T_e$ (below the thermoneutral zone for this species; Ref. 13) was chosen, since it was previously shown to support hypothermia and fever development and is essential for heat stroke recovery in this species (20).

Mice were assigned to one of the following groups for blood and tissue collection: 1) baseline, 2) $T_{e,\text{Max}}$, 3) hypothermia, or 4) 24 h of recovery. Criteria for inclusion in each sampling group are shown in Table 1. Figure 1 depicts the relationship of these group designations to the 48-h $T_e$ response of a mouse during progression to and recovery from heat strain. Baseline was defined as $T_e < 36.0°C$, which represents the daytime (inactive period; 12-h average) $T_e$ nadir in this species (20). Baseline samples were collected between 0900 and 1000 (2–3 h after lights on) to avoid circadian influences on the $T_e$ response during heat exposure. $T_{e,\text{Max}}$ was equivalent to 42.7°C. This level of...
lowest Tc observed during recovery at Ta of 25°C (Fig. 1). It was stress (dashed line) C57BL/6J male mouse during 48 h of experimentation. sampling as it relates to the thermoregulatory profile of each mouse. Tc and

5 min, until plasma could be separated by centrifugation (room temperature; heparinized microcentrifuge tubes and immediately placed onto ice

ized 1-ml syringe, 23-gauge needle); blood was placed into 1.5-ml

guinated following thoracotomy and intracardiac puncture (heparin-

, IL-2, IL-4, IL-6, IL-10, IL-12p40, IL-12p70, IFN-

The FlowMetrix System is a “Multiplexed Fluorescent Bead-Based which permits the simultaneous quantitation of multiple cytokines. A custom 11-plex kit (Bio-Rad Laboratories, Her-
removal from the heat and placement into a 25°C environment, mice develop hypothermia followed by a feverlike Tc elevation from ~24–36 h of recovery (Fig. 1; Ref. 20).

Mice of the heat stress group required 252 ± 8 min to reach Tc,Max of 42.7°C, which was equivalent to a heating rate of 0.034 ± 0.003°C/min (data not shown). These conditions imposed a total thermal load (thermal area) of 279.4 ± 9.5°C-min and induced ~10% dehydration in heat-stressed mice. Control mice experienced ~2% dehydration, presumably due to the absence of food and water during experimentation and stress-induced hyperthermia in response to the weighing procedure for BW determination (20). Table 1 provides the Tc values observed in each control and heat stress group. A hypothermia depth of 29.3 ± 0.4°C was observed in heat stress mice at ~115 min after removal from the heat (i.e., at 369 ± 24 min of recovery), which was significantly lower than that in control mice (36.4 ± 0.4°C; P < 0.001). At ~24 h the following day, Tc of heat stress mice was 37.8 ± 0.3°C, which represented a significant Tc elevation compared with nonheated controls (36.2 ± 0.3°C; P < 0.001).

Plasma cytokine response. IL-1α, IL-2, IL-4, IL-12p70, IFN-γ, TNF-α, and MIP-1α levels did not show a significant elevation above baseline at any time point in the control or heat stress condition (data not shown). The four cytokines, which were significantly elevated at different times in the heat stress condition, included IL-1β, IL-6, IL-10, and IL-12p40 (Fig. 2).

IL-1β. Plasma IL-1β levels were observed at low levels (4–5 pg/ml) in control mice at all time points, indicating that 1) our blood collection protocol did not induce cytokines in the absence of heat stress, and 2) there is not a circadian variation to the production of IL-1β under the conditions of our experimental protocol. We did not detect a circadian variation in the production of any cytokine measured in this study (Fig. 2 and data not shown). Heat stress mice showed a significant elevation in IL-1β levels compared with controls at hypothermia (14.1 ± 3.0 vs. 5.2 ± 2.1 pg/ml; P = 0.027). Although IL-1β levels appeared to be decreased compared with controls at 24 h of recovery, this did not represent a significant difference (P = 0.173), such that IL-1β levels were equivalent to baseline at this time.

IL-6. Plasma IL-6 levels were below the limit of assay detection at all time points in nonheated controls (Fig. 2B). IL-6 production in heat stress mice showed a tendency toward increased levels at Tc,Max, but this did not represent a significant difference from controls (P = 0.55). Heat stress mice showed a significant increase in IL-6 production at hypothermia (199.1 ± 53.8 pg/ml; P = 0.001). This was the largest cytokine increase induced by heat exposure. IL-6 remained elevated in heat stress mice compared with controls at 24 h of recovery (45.0 ± 17.4 pg/ml; P = 0.026), although the levels had diminished from that observed at hypothermia (Fig. 2B).

IL-10. IL-10 was measured at low basal levels at all time points in control mice (3–5 pg/ml; Fig. 2C). Heat stress mice showed a significant elevation in IL-10 at hypothermia compared with controls (160.2 ± 59.1 vs. 2.8 ± 0.9 pg/ml; P = 0.014). At 24 h of recovery, IL-10 levels had returned to baseline and were indistinguishable from those observed in control mice (3.0 ± 1.2 vs. 4.9 ± 1.5 pg/ml; P = 0.325).

IL-12p40. IL-12p40 showed the highest baseline levels at all time points (43–55 pg/ml; Fig. 2D). IL-12p40 was the only cytokine that showed a significant elevation above control levels at Tc,Max (79.8 ± 6.8 vs. 43.4 ± 4.4 pg/ml; P < 0.001). IL-12p40 levels were similar to those of controls at hypothermia, but they had significantly decreased below control levels by 24 h of recovery (43.3 ± 8.9 vs. 19.7 ± 2.4 pg/ml, P = 0.019; Fig. 2D).

Fig. 2. Plasma concentrations of IL-1β (A), IL-6 (B), IL-10 (C), and IL-12p40 (D) at several time points during recovery in C57BL/6J mice exposed to the control or severe heat strain condition. Details of each group are depicted in Fig. 1 and described in Table 1. Sample size was 8–11 mice/group. Values are means ± SE. Differences between control and heat stress mice were examined at each sampling time point, with significance set at P < 0.05.
Plasma corticosterone response. Figure 3 shows plasma corticosterone levels in control and heat stress mice. Baseline corticosterone levels were 9.1 ± 1.8 ng/ml, which was indicative of a resting, quiescent state before heat stress experimentation. The absence of a significant corticosterone response in control mice indicates that the blood collection protocol (e.g., anesthesia) did not induce a stress response in the absence of heat exposure and thus was not a confounding stressor in our model. Circadian corticosterone displayed a circadian oscillation in control mice with significantly increased levels at Tc,Max and hypothermia compared with baseline (Fig. 3; P = 0.003 and P < 0.001, respectively). These time points corresponded to ~6 h (1300) and ~3.5 h (1530) before the lights-off (1900) or active period for this species. Heat stress mice showed a significant increase in plasma corticosterone at Tc,Max and hypothermia compared with controls (P < 0.001; Fig. 3); the levels observed at hypothermia were significantly greater than those observed at Tc,Max (348.3 ± 38.8 vs. 215.9 ± 35.5 ng/ml; P = 0.024). By 24 h of recovery, corticosterone levels of heat stress mice had returned to baseline and were virtually indistinguishable from those observed in the control group.

Correlation analysis. IL-1β, IL-6, and IL-10 showed a significant (inverse) correlation with Tc (r = 0.40, P = 0.004; r = 0.55, P = 0.001; r = 0.56, P = 0.001, respectively; Fig. 4A). IL-12p40 and corticosterone did not correlate with Tc (r = 0.04, P = 0.082; r = 0.31, P = 0.117, respectively; data not shown). Positive correlations were detected for IL-6 and IL-10 compared with IL-1β (r = 0.55, P = 0.001; r = 0.82, P < 0.001, respectively; Fig. 4B) for IL-1β and IL-10 compared with IL-12p40 (r = 0.59, P < 0.001; r = 0.54, P < 0.001, respectively; Fig. 4C) and for IL-10 compared with IL-6 (r = 0.55, P = 0.001; Fig. 4D). IL-6 has been strongly implicated as a modulator of the hypothalamic-pituitary-adrenal (HPA) axis; corticosterone and IL-6 were positively correlated at a borderline level of significance (r = 0.38, P = 0.052; Fig. 4D).

Hematocrit and glucose. Hematocrit ranged from 35 to 37% in control mice at all time points (Table 2). Values showed a tendency to increase in heat stress mice at Tc,Max but this did not represent a significance difference from controls (Table 2). This finding was surprising since we calculated ~10% dehydration at this time point from our BW measurements (data not shown). At hypothermia, hematocrit values were significantly elevated in heat stress mice compared with controls (48 ± 2 vs. 35 ± 1%; P < 0.05; Table 2). By 24 h of recovery, hematocrit concentrations had returned to baseline levels and were virtually indistinguishable between heat stress and control groups.

Plasma glucose levels ranged from ~140–180 mg/dl in control mice (Table 2). A tendency toward increased glucose levels in control mice at the Tc,Max (~1300) collection time point was not representative of a significant change from baseline (P = 0.55). Hypoglycemia (~65–75% decrease) was evident in heat stress mice at Tc,Max and hypothermia, with recovery to baseline by 24 h (Table 2).

Tissue histopathology. Tissue samples were collected from a different set of animals than those used for plasma determinations; however, total thermal load (289.9 ± 10.6°C·min) and dehydration (~10%) were virtually identical between mouse populations. No significant abnormalities were detected in control mice. Histopathology was performed on eight tissues, with lesions in heat stress mice detected in the kidney, spleen, and small intestine only (Table 3). No significant abnormalities were detected in liver, heart, lung, or large intestine of any mouse. Representative photomicrographs of renal, splenic, and small intestine lesions observed at different phases of recovery are shown in Fig. 5. Renal tubular necrosis was observed primarily in the straight tubules of the lower cortex, as characterized by shrunken, acidophilic, and fragmented epithelial cells with pyknotic nuclei (Fig. 5B). Splenic damage was initially detected at hypothermia, with the number of lesions decreasing by 24 h of recovery (Table 3). Lymphoid necrosis was observed in the white pulp of the spleen, characterized by small clusters of nuclear and cellular debris that each occurred within a tingible body macrophage (Fig. 5D). Mild incidence of lesions in the small intestine were detected at Tc,Max and showed a diminution in incidence throughout recovery (Table 3). Intestinal lesions were characterized by exfoliated enterocytes and dilation of central lacteals of the villi (Fig. 5E).

DISCUSSION

Several pro- (e.g., IL-1β) and anti-inflammatory (e.g., IL-10) cytokines are observed at elevated levels in the circulation of heatstroke patients (2–4, 9). Due to the determination of circulating cytokine levels primarily at end-stage heatstroke or at the time of clinical presentation (often after cooling has occurred), our understanding of Tc-dependent changes in the balance of these mediators during long-term progression of the heat syndrome remains poorly understood. We present here the first study, using a mouse model, to determine changes in the circulating levels of 11 cytokines across several days of the heat syndrome and correlate these changes with distinct Tc phases in conscious, undisturbed animals. We showed that plasma concentrations of IL-1α, IL-2, IL-4, IL-12p70, IFN-γ, TNF-α, and MIP-1α were unaffected by the level of heat strain imposed in our model, whereas IL-1β, IL-6, IL-10, and IL-12p40 were increased in a time- and Tc-dependent manner.
While IL-12p40 was the only cytokine observed at $T_{c,\text{Max}}$, IL-1$\beta$, IL-6, and IL-10 levels were maximally elevated at the depth of hypothermia. Corticosterone, which is a natural inhibitor of cytokine production, was significantly elevated from $T_{c,\text{Max}}$ to hypothermia and returned to baseline levels by 24 h of recovery. Interestingly, IL-6 was the only cytokine elevated at 24 h after heat exposure, when a feverlike $T_c$ elevation was observed.

An important finding in our study was the absence of increased circulating cytokine and corticosterone levels in nonheated control mice subjected to the plasma and tissue collection protocol. This demonstrates the appropriateness of the use of conscious, freely ranging animals for the study of heat-induced thermoregulatory, hemodynamic, and tissue injury responses. Previous rodent models of heat stress have been compromised by the use of restraint, rectal probes, and/or anesthesia for the measurement of heat-induced responses (12, 21, 22, 30, 33). These methodologies prevent natural behavioral adaptations to heat stress (e.g., salivary spreading), alter thermoregulatory control mechanisms, and induce stress-in-
duced increases in cytokine and corticosterone production that confound data interpretation. For example, in rats, anesthesia was shown to induce IL-1β production in the absence of heat stress (21). By using biotelemetry for the continuous measurement of Tc in freely moving conscious mice, these confounders have been eliminated, allowing a more stringent measure of the time course of effects of heat exposure on pathophysiological responses. Similarities between findings in heat stress mice and those previously described for human heatstroke cases support the applicability of our model for the study of cytokine-induced pathophysiological responses in vivo.

Surprisingly, we did not observe increased IL-1β, IL-6, or IL-10 levels at Tc,Max, despite several studies showing elevations in the circulating levels of these cytokines at end-stage heatstroke (2–4, 6, 21, 25). There are two potential reasons for this finding; first, previous studies defined end-stage heatstroke as the time point at which a severe reduction in mean arterial pressure (MAP) was observed (21, 22), whereas changes in MAP were not determined in our study. Thus Tc,Max may not be representative of end-stage heatstroke (i.e., MAP reduction) in our model. Rather, the observation of maximal levels of IL-1β, IL-6, and IL-10 at hypothermia depth, along with the occurrence of respiratory depression and bradycardia (personal observations during blood collection procedures), suggests that this time point is more indicative of end-stage heatstroke (i.e., circulatory collapse) in our model. Second, the level of heat strain imposed by our protocol is associated with ~8% mortality (20), whereas previous studies are more representative of a non-survival protocol (100% mortality; Refs. 21, 22).

Table 2. Hematocrit and plasma glucose response to severe heat strain

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Tc,Max</th>
<th>Hypothermia</th>
<th>24-h Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hematocrit, %</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>37 ± 2 (3)</td>
<td>37 ± 1 (4)</td>
<td>35 ± 1 (5)*</td>
<td>36 ± 1 (6)</td>
</tr>
<tr>
<td>Heat</td>
<td>39 ± 3 (4)</td>
<td>48 ± 2 (5)*</td>
<td></td>
<td>35 ± 2 (7)</td>
</tr>
<tr>
<td><strong>Glucose, mg/dl</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>139.5 ± 7.8 (9)</td>
<td>181.6 ± 9.6 (7)*</td>
<td>165.2 ± 12.6 (8)*</td>
<td>166.6 ± 9.9 (11)</td>
</tr>
<tr>
<td>Heat</td>
<td>43.6 ± 16.8 (6)*</td>
<td>56.4 ± 16.0 (5)*</td>
<td></td>
<td>173.9 ± 17.7 (7)</td>
</tr>
</tbody>
</table>

Values are means ± SE. Sample sizes are indicated in parentheses. Group criteria are described in Table 1. *Significant difference (P < 0.05) between control and heat stress condition within a sampling group.

Table 3. Incidence of lesions in hematoxylin-eosin-stained mouse tissues

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Tc,Max</th>
<th>Hypothermia</th>
<th>24-h Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Renal cortical tubular necrosis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimal</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Mild</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Moderate</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Splenic lymphoid necrosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimal</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Mild</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Moderate</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Small intestine</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Minimal</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Mild</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Moderate</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

n. Sample sizes (in parentheses). Group criteria are described in Table 1. Number indicates total number of animals in which abnormalities were detected. ND, not detectable. See MATERIALS AND METHODS regarding criteria for injury assessment.

The presence of high plasma IL-12p40 levels at Tc,Max represents the first demonstration of increased circulating levels of this cytokine in an experimental heat stress model. IL-12p40 is also known as the IL-12 receptor antagonist, as it binds to the IL-12 receptor, but fails to induce a signal (28). Thus it functions as a natural inhibitor of IL-12p70 (agonist also referred to as IL-12) function. IL-12p70 production is sensitive to pathogenic stimulation (7). The implication of enhanced endotoxin release in patients and animal models of heatstroke (2, 12, 14, 17) led us to hypothesize that increased IL-12p70 levels may be observed in mice during heat strain recovery; however, IL-12p70 was not observed at any time point. It is unclear if we missed the time point of heat-induced IL-12p70 release, if it was produced locally such that it did not appear in the systemic circulation, or if its production was not stimulated by the level of heat strain imposed in our mouse model (e.g., endotoxin levels were not determined). The lack of correlation between IL-12p40 and Tc levels suggests that production of this receptor antagonist is dissociated from the robust Tc changes observed in our model.

During heat strain recovery, mice displayed a biphasic thermoregulatory response consisting of initial hypothermia (~29°C) and subsequent feverlike Tc elevation (~1.5°C) the day following heat exposure. Although the magnitude of the response varies widely between studies, heat-induced hypothermia has been observed in several species, including guinea pigs (30), mice (20, 33), and rats (24). While heat-induced hypothermia is thought to be important in recovery and survival from life-threatening Tc elevations (20, 33), little is
known regarding the physiological mechanisms regulating this response. Chang (9) reported the ability to cool heatstroke patients from 40 to 38°C to be dependent on serum IL-1β levels, which is the only report demonstrating a role for an endogenous cytokine in core cooling with heatstroke. While hypothermia is a natural recovery response to heatstroke in experimental animals (20, 24, 30, 33), it has not been observed in heatstroke patients. Whether this is a consequence of body scaling issues (i.e., smaller surface area-to-body mass ratio of humans that does not support rapid dissipation of core heat to the environment) or clinical interventions that have masked the response is unclear. However, the association of hypothermia with mitigation of heat-induced intestinal damage and enhanced survival in mice (20) suggests that cooling of heatstroke patients to a hypothermic level (i.e., \( T_c < 37^\circ C \)) may be beneficial for the prevention of tissue (e.g., central nervous system) injury in this syndrome. Support for this contention is provided by the use of induced hypothermia, in which \( T_c \) is physically decreased using cooling blankets or other methods, as a protective measure during cardiopulmonary bypass surgery and as treatment for cerebral ischemia and stroke (11, 27). The contention that hypothermic treatment would be more efficacious if regulated, rather than forced reductions in \( T_c \) were implemented, suggests that further studies are required to determine the regulated nature of hypothermia under injurious conditions.

Our study represents the first report to show increased plasma concentrations of IL-1β, IL-6, and IL-10 at the depth of hypothermia during heat strain recovery. It is currently unclear in our model if cytokines are inducing hypothermia or vice versa. The presence of increased cytokine concentrations before heat-induced hypothermia would have been more infor-
mative regarding a potential causal relationship between these phenomena, indicating that future studies would benefit from an examination of plasma cytokine levels during the cooling phase of recovery (e.g., after return to baseline). Although we were able to detect a significant inverse correlation between several cytokines and $T_c$ (Fig. 4), these results do not indicate causation with respect to cytokines driving the observed $T_c$ responses. Ultimately neutralization studies using antibodies or gene knockout models will be required to delineate the role of individual cytokines, such as IL-12p40, in heat-induced hyperthermic $T_c$ responses.

In humans, fever is a symptom of heatstroke, persisting for 7–14 days following clinical presentation in some patients (26). We hypothesize that the $T_c$ elevation observed at 24 h of recovery is a fever that is regulated by endogenous IL-6, which was elevated both before and at this time point in our model (Fig. 2). Fever is defined as a regulated increase in the thermal set point and is a common manifestation of SIRS in disease and injurious states. While a protective effect of fever in infection is recognized (16), its role in heat-induced SIRS is unknown. IL-6 has been strongly implicated as an endogenous pyrogen (i.e., fever inducer) based on production of fever following its injection, increased circulating levels during fever, and the absence of fever under conditions in which its endogenous actions are neutralized (16, 18). IL-6 is commonly observed at elevated levels in heatstroke patients and shows the highest correlation with mortality and neurological symptoms of heatstroke, thus implicating it as a potential therapeutic target for heatstroke treatment strategies (3, 6). It would be interesting to determine the effect of IL-6 neutralization or antipyretic (e.g., nonsteroidal anti-inflammatory drugs) treatment on mortality and the 24-h feverlike $T_c$ elevation in our heat strain model.

Glucocorticoid hormones (corticosterone in rodents and cortisol in humans) are produced by the adrenal gland and represent an integral component of the HPA axis. In addition to the mobilization of energy stores during stress, glucocorticoids act as natural inhibitors of cytokine production in response to stress. We observed a direct correlation between IL-6 and corticosterone, suggesting that this cytokine is activating the HPA axis during the heat syndrome. Classical heat stress induces significant increases in cortisol secretion in heatstroke patients (1). A protective effect of dexamethasone, a synthetic glucocorticoid, in the amelioration of heatstroke symptoms, such as hypotension, cerebral ischemia, and neuronal damage in rats, was correlated with a reduction in endogenous IL-1β levels (23). Thus interactions between endogenous cytokines, such as IL-1 and IL-6, with components of the HPA axis appear to be modulating several of the pathophysiological responses observed during the heat syndrome.

We observed a variety of pathophysiological changes in response to prolonged heat exposure, including dehydration, hypoglycemia, and tissue injury that may be responsible for the $T_c$ changes and enhanced cytokine production observed during heat strain recovery. In rodents, the major heat loss mechanism during heat exposure is the behavioral spreading of saliva and/or urine onto highly vascularized body surfaces to facilitate evaporative cooling. Our heat stress protocol prevented food and water consumption during ~4 h of heat exposure, which induced ~10% dehydration (as determined by BW measurements at $T_{c, Max}$). The occurrence of renal damage may have also been related to heat-induced dehydration in our model. Interestingly, these changes were observed with a concurrent reduction (~76%) in plasma glucose levels. Hypoglycemia was not an unexpected observation in heat-stressed mice, given the high metabolic demands of prolonged heat exposure, in the absence of food and water intake, and the report of this phenomenon in other experimental heat stress models (6). While cytokines, such as IL-1 and IL-6, are capable of inducing hypoglycemia (29, 31), the presence of significant hypoglycemia at $T_{c, Max}$ (i.e., before increased production of IL-1 and IL-6) suggests that heat exposure was responsible for this response in our study. An absence of liver damage in heat stress mice suggests that thermal injury to this organ was not responsible for hypoglycemic responses in heat-stressed mice. Previous reports showing an ability of dehydration and hypoglycemia (or food restriction) to induce hypothermia in small rodents suggests that these pathophysiological changes represent two, of perhaps several, physiological stimuli driving hypothermia development in heat-stressed mice (8, 15).

In heat shock, circulatory adjustments that shunt core blood from the splanchnic organ bed to the skin for heat dissipation induce intestinal damage, resulting in increased permeability of the epithelial barrier and translocation of endotoxin from the gut lumen to the circulation (12, 14, 17). Although we did not measure circulating endotoxin levels in heat-stressed mice, pathological examination of the small intestine revealed lesions to this organ that are similar to those reported in heatstroke cases in which endotoxemia has been observed (14, 17). Increased circulating endotoxin concentrations have been observed in primates and rodents models of heatstroke at $T_c$ as low as 39–40 or 41.5–42°C, respectively (12, 17). Several of the responses in the present study during heat strain recovery are similar to those observed in response to endotoxin, including increased cytokine and corticosterone production, tissue injury, hypoglycemia, and a biphasic thermoregulatory response consisting of hypothermia and fever (16, 18, 29, 31).

Tissue damage is a common manifestation of the heatstroke syndrome (6, 10, 17). In heat-stressed mice, the time course of the incidence of lesions to the small intestine, spleen, and kidney differed throughout 24 h of recovery. Whereas the small intestine was maximally damaged at $T_{c, Max}$, the highest incidence of splenic lesions was observed at hypothermia depth. Interestingly, thermal injury to these tissues appeared to be resolving as animals progressed through 24 h of recovery. On the other hand, renal damage, which is almost universally observed in heatstroke patients, showed increased incidence of damage throughout recovery (Table 3). These data suggest that long-term monitoring of tissue injury (i.e., beyond 24 h) may have revealed progressive renal damage as well as damage to additional organs that appeared normal in this study. For example, liver damage is primarily observed in long-term survivors of heatstroke (26). Whether damage to the small intestine, spleen, or kidney was a consequence of direct thermal damage or SIRS-induced pathophysiological changes cannot be determined from this study. However, it does raise the issue of the importance of correlating changes in circulating cytokine levels with those observed at the tissue level. For example, it is important to note that changes in circulating concentrations of any cytokine may not be representative of changes occurring at the tissue level, the latter of which are likely more important for the direct effects of these substances.
on heat-induced pathophysiological responses. Ultimately, circulating biomarkers are the measures that are most easily obtainable in humans in a clinical and/or field setting; thus it will be important in future studies to determine cytokine levels in those organs that showed tissue damage in mice during heat strain recovery and to correlate tissue and circulating levels of these substances, an endeavor that will likely be aided by the application of genomic or proteomic technologies.

ACKNOWLEDGMENTS

We thank L. Walker, J. Castor, and M. Valliere for invaluable technical assistance with heat stress experimentation and plasma/tissue collection, and R. Wallace for assistance with statistical analysis.

DISCLAIMER

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Army or the Department of Defense.

In conducting the research described in this report, the investigators adhered to the “Guide for Care and Use of Laboratory Animals” as prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

REFERENCES


