Biomarkers of multiorgan injury in a preclinical model of exertional heat stroke

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EXERTIONAL HEAT STROKE (EHS) and related exertional heat injuries can occur when there is an inability to adequately dissipate the heat load induced by physical exertion, resulting in extreme elevations in core temperature and damage to multiple organ systems (10). Although EHS is more likely to occur in hot and humid environments, individuals can be at risk over a wide range of environmental conditions (57). EHS also can occur in seemingly healthy individuals compared with passive heat stroke (PHS), which often transpires in subjects who are very young or elderly or have significant comorbidities (23). Recent data demonstrate that hospitalizations due to EHS and related exertional heat injuries are frequent and have been on the rise in the U.S. civilian population (51). EHS is considered the third leading cause of sudden death in high school athletes (37) and the U.S. armed forces experienced 323 cases of hospitalization due to EHS in 2013, with greater than 1,710 cases of heat injury, most of which are associated with exertion in the heat (4).

To date, there have been a limited number of published preclinical animal models of EHS (16, 33, 34). Although important findings have arisen from existing models, particularly from Hubbard et al. in a rat model (32, 33, 35), the existing approaches prove to be unsatisfactory as they have significant limitations, including superimposed stress responses induced by shock avoidance, stressful types of instrumentation such as rectal thermistors, and very high mortality rates. Other models have stopped at predefined maximum core temperatures (Tc,max) that are more likely to induce heat injury rather than heat stroke (16). In contrast, animal models of PHS in unrestrained and unanesthetized rodents have been well developed (46, 47, 56). PHS in mice results in a characteristic thermoregulatory profile (46) and displays disorders of the central nervous system, hepatic, renal, gastrointestinal, and musculoskeletal systems (45, 47). However, the extent to which PHS models translate to conditions of EHS is unknown, making parallel development of models of EHS essential for understanding mechanisms, for understanding comorbidities, or for developing new preventative strategies or treatment options. Although human studies of the physiology of exercise in hyperthermia provide valuable information regarding adaptive mechanisms, they can only go part way to understanding underlying pathophysiology of this life-threatening EHS condition.

EHS is of particular importance to study because it is the predominant form of heatstroke in the military, athletes, and occupational workers. Theoretical differences also exist in the pathophysiology of EHS compared with PHS. In previous models in the rat, Hubbard et al. (35) showed that EHS has a greater impact on multiple organ injury at lower core temperatures, compared with PHS. During exposure to hyperthermia, the shunting of blood towards the body surface for heat exchange can lead to underperfusion of splanchic vascular beds and is likely to involve a greater degree of ischemic injury rather than thermal injury to the intestine and other internal organs (42). This is potentiated during exercise in the heat because additional cardiac output is shunted to the exercising muscles (59). These and other integrative physiological interactions are also likely to promote brain hypoperfusion and facilitate the onset of severe neurological symptoms, one of the specific hallmarks that distinguishes heat stroke from heat injury (3).

Exercise also presents an additional stress on metabolic pathways. Heat alone increases glucose utilization (60), and exercise in the heat can pose an even greater challenge to sustaining metabolic substrates for brain, heart, and muscle
function. EHS induces rhabdomyolysis, which is rarely seen in PHS. The susceptibility of muscle to rhabdomyolysis is greatly enhanced during heat exposure (5). Rhabdomyolysis contributes to acute kidney failure through secondary reactions of myoglobin released into the circulation (62), and it can promote coagulopathy, believed to occur from release of tissue clotting factors from damaged muscle (66). Thus the additional stressors that exercise presents during a hyperthermic challenge elevate the potential for multiple organ injury and dysfunction in EHS.

In this study we report a new model of EHS in the mouse that carries with it many of the pathophysiological characteristics seen in humans. Our primary purpose was to create a tool for future development of treatment and prevention strategies that directly apply to humans with EHS. We also determined 1) the relative impact of a range of different temperatures and humidities on the characteristic EHS response in mice, 2) the typical core temperature profile observed in response to EHS and recovery over 24 h, and 3) the extent and range of multiple organ injury and metabolic disorders induced by EHS. We demonstrate that many pathophysiological characteristics of EHS in mice are similar to those observed in mice exposed to PHS, including multiorgan injury. However, we demonstrate that EHS is highly distinguishable from PHS due to the profound neurological symptoms at the maximum core temperatures achieved, extensive rhabdomyolysis and injury over the entire expanse of the small intestine.

METHODS

All animal protocols were approved by the University of Florida (UF) Institutional Animal Care and Use Committee. Because of the stressful nature of inducing heat stroke in animals, the early pilot experiments were monitored carefully in real time by the UF Veterinary Medical Staff and met their approval criteria for humane animal testing. Eighty-one mice were used for data collection in this study. All were C57BL/6J males (Jackson Laboratories, Bar Harbor, ME) weighing an average of 30.2 ± 2 (SD) g with an approximate age of 4 mo. They were housed in groups until they were implanted with telemetry devices, after which they were individually housed in 7.25-in. wide × 11.75-in. diameter × 5-in. high cages, maintained on a 12:12-h light cycle at 20–22°C and 30 – 60% relative humidity (RH). A standard chow diet and water were provided ad libitum until the EHS protocol. Experiments were performed in the morning of the light cycle.

Animal preparation and training. While they were under isoflurane anesthesia, mice were implanted with temperature telemetry transmitters (TA-E-Mitter; Starr Life Sciences, Oakmont, PA), placed in their abdominal cavities for real time monitoring of core temperature (Tc). The mice were then allowed to recover with subcutaneous buprenorphine injections, every 12 h for 48 h and then monitored for a minimum of 2 wk. At that time, exercise wheels and enrichment huts (Silent Spinner and Small Animal Igloo Hideaways; Petco, San Diego, CA) were introduced into the cages for 3 wk. During this period, mice had ad libitum access to the running wheel throughout the day and night. On the 3rd week, additional exercise training was implemented to familiarize the mice to the environmental chamber in the laboratory (Thermo-Forma 3940 Incubator; Thermo-Fisher, Waltham, MA) and to the customized running wheel system (Lafayette Model 80840, Lafayette, IN). The forced running wheel was adapted to operate directly via a manual power supply, and the positions of the running wheel (4 cm lower) and motor (15 cm higher) were altered to eliminate interference from the wheel motor and improve the fidelity of the E-Mitter signal. The wheel speed was continuously monitored. The first exercise session consisted of 15 min of free-wheeling where the mouse was free to run in the spiked wheel without a forced pace and explore their surroundings. This was followed by a short recovery period (<5 min). Then, mice were started at an initial speed of 2.5 m/min, which was increased by 0.3 m/min, every 10 min, for 60 min. Training sessions on the next 2 consecutive days consisted of only the incremental protocol on the forced wheel, lasting for 60 min each. The fourth and final session used the same protocol; however, exercise time and incremental speed were elevated until the animals exhibited fatigue. Fatigue was defined as refusal to run or walk with the wheel for >5 s. No shock or any other manual stimuli were used to maintain running speed.

Exertional heat stroke. Following the last training session, mice were given 2 days of rest, with free access to the running wheel in their cage. The following morning they were brought to the laboratory in their own cage and allowed to rest for a minimum of 2 h while Tc was monitored. A data acquisition system was used to collect continuous Tc, averaged over 30-s intervals (VitalView; Starr Life Sciences). After at least 2 h of resting data in the environmental chamber, each mouse was monitored until Tc dropped to <37.5°C for >15 min. Laboratory and environmental chamber conditions were kept constant during this time, similar to animal housing facilities. Then, the environmental temperature (Tenv) and RH were increased to the target values. As soon as the environmental chamber equilibrated to the target Tenv, the chamber was opened and the animal quickly placed in the running wheel. The forced running wheel protocol was then initiated. The mouse’s behavior was monitored continuously with a video camera. Running speed began at 2.5 m/min and increased 0.3 m/min every 10 min until the mouse reached a Tc of 41°C, which served as a threshold beyond which the speed was kept at a steady state for the remainder of the protocol. The study design considered 42.5°C as a humane end point. This end point was selected based on the minimum threshold temperature that induces symptoms of heat stroke in previous studies using an established PHS model (46). At the end of the protocol, Tenv was adjusted back to room temperature, the chamber door was opened, and the mouse was carefully watched until it regained consciousness. At this time, it was weighed and returned to its cage, while Tc continued to be monitored for 24 h of recovery or until death at an earlier time point. The 12-h light-dark cycle was maintained in the laboratory during the recovery period.

In each experiment, "thermal area" was used as an estimate of "thermal stress" or "thermal dose" and calculated as defined by Leon et al. (46). Mathematically this equals approximately the area under the curve of the temperature profile for all points at which Tc was >39.5°C (units = °C·min). The baseline reference Tc (39.5°C) was chosen because in PHS models (e.g., as used in the control PHS in this study, described below) the chamber Tenv is maintained at 39.5°C. Therefore, this is a point where mice are unable to remove excess heat by radiation alone. In this current study we used Tenv ranging from 37.5 to 39.5°C for EHS but kept the 39.5°C as the cut off for comparison of thermal area results between PHS and EHS at the various environmental temperatures.

Experimental series 1. The first series of experiments were designed to study the Tc profiles and survival of mice exposed over a range of Tenv and RH values during exercise. Thirty mice were studied in series 1, with five groups of six mice per group. Three groups were exposed to 37.5, 38.5, or 39.5°C at 50% RH and two groups were exposed to 30 or 90% RH, studied at 37.5°C. After 24 h, all mice were returned to the UF Animal Facility in their own cages, where they were allowed to recover for a total of 4 days, to evaluate short-term survival. After 4 days, animals were placed under isoflurane anesthesia and blood samples were obtained by transthoracic cardiac stick. Soleus, gastrocnemius, diaphragm, heart, liver, kidney, spleen, intestine, and brain were removed for later biochemical or histological analyses. Animals were exsanguinated and a thoracotomy with heart removal was performed for euthanasia under deep anesthesia.

Experimental series 2. Following the establishment of EHS model and the effects of changes in Tenv and RH, three groups of additional
mice were studied (n = 6–8 per group) to determine the time course of multorgan injury. All groups in series 2 experienced EHS at 37.5°C at 50% RH and were killed at either 0.5, 3, or 24 h after EHS for blood and tissue sample collection. Three other groups of sham (fully instrumented and trained) exercise control mice (EXC) were killed at the same time periods after undergoing matched intensities and durations of exercise (max speed: 5.2 m/min, duration: 113 min) at 25°C and 50% RH.

**Experimental series 3.** Tissue and blood samples from a second control group of mice that did not undergo any exercise training or any interventions (n = 6) served as untreated mouse controls, i.e., naïve controls (NC). Another group of animals was also studied in this series, which were exposed to a PHS protocol (n = 6). These mice were exposed to 39.5°C at 30% RH, similar to previous approaches described by Leon et al. (46). However, to mimic the heat exposure in the 37°C/50% RH EHS group in series 1 and 2, mice stopped after reaching a \( T_{c,max} \) of only 42.1°C. Because extensive data have been collected in similar models of PHS, we studied only this single time point, 3 h after reaching \( T_{c,max} \), which corresponds to a time where there is considerable evidence of organ injury.

**Biochemical analyses.** Blood was collected with heparin and spun at 2,000 RCF and plasma (250 \( \mu L \)) was taken from the sample and stored at −80°C. Plasma was sent to UF College of Veterinary Medicine Diagnostic Laboratories for determination blood urea nitrogen (BUN), creatinine, alanine transaminase (ALT), and creatine kinase (CK) (Siemens Dimension Xpand Plus Integrated Chemistry System).

**Analysis of histological samples.** The procedure for obtaining and grading of fixed histological samples of the small intestine has been previously described (52) and follows the method derived from Chiu and Quaedackers (14, 55). Briefly, 1-cm transverse sections from the duodenum, jejunum, and ileum were taken for cutting and hematoxylin-eosin staining at the UF College of Medicine Clinical Pathology Laboratory, with a section thickness of 4 \( \mu \)m. Histology slides were then graded and modified to apply to randomly selected individual villi. Two trained and blinded raters separately graded samples from each slide. The average score per sample across raters was reported. Villus height and width were measured using calibrated microscope image analyses. In some tissues a small number of fixed samples from randomly selected control and EHS animals were used for qualitative histopathological assessment. These were taken at time points where the corresponding biomarkers suggesting injury had peaked.

**ELISA.** Plasma ELISA kits for detection of fatty acid binding protein 2, intestinal (FABP2) were purchased from USCN Life Science (Wuhan, Hubei). Briefly, a microtiter plate provided with the kit was precoated with an antibody specific to FABP2. Plasma samples were added to the wells with a biotin-conjugated antibody specific to FABP2. Then, avidin conjugated to horseradish peroxidase was added to each well and incubated. After a TMB substrate solution was added, wells that contained FABP2 biotin-conjugated antibody and enzyme-conjugated avidin exhibited a change in color proportional to the amount of FABP2 present. Color change was measured spectrophotometrically at a wavelength of 450 nm. The concentration of FABP2 in the samples is determined by comparing the optical density of the samples to the standard curve using Microsoft Excel.

**Plasma glucose.** Blood glucose was obtained at the time of death using a handheld glucose meter (OneTouch, Lifescan).

**Statistical analyses.** Statistical analyses were performed using SAS JMP (Cary, NC). One-way ANOVA was used to determine differences between groups, with post hoc Tukey’s test when data were parametric. Kruskal-Wallis or Wilcoxon were used for nonparametric comparisons with central tendency expressed as medians ± 25–75% quartiles. Small durations of noise in the \( T_{c} \) data collected from the transmitters were considered an artifact due to transmitter reception difficulties (a rare event) and were treated as missing data and extrapolated over the appropriate interval. Where appropriate, effects of multiple sampling were determined by using comparisons that met an acceptable false discovery rate (FDR = 0.15) by performing the Benjamini-Hochberg procedure (8).

**RESULTS**

**Effects of environmental temperature.** All animals that were followed over the 4-day recovery period survived the EHS protocol, and all animals that were killed at intermediate time points survived up to their targeted end points. At 24 h, the animals were categorized as to their level of brightness, alertness, and responsiveness. There were no signs of morbidity (righting reflex) after the first few hours of recovery. Body weights returned or exceeded pre-EHS levels by 4 days. The exercise-hyperthermia protocol was stopped in all but 3/42 EHS animals because of loss of consciousness or stupor that was indistinguishable from loss of consciousness. This was characterized by the mice laying on their back or side, without the ability or willingness to right themselves and by being unresponsive to tactile stimuli. After the short period of unconsciousness of <5 min, the animals quickly recovered upright mobility, a return to responsiveness to gentle tactile stimuli and a return to quiet resting and grooming behavior in their home cage. The other three mice reached the humane end point of a \( T_{c,max} = 42.5°C \), based on previous end points in a PHS models in mice by Leon et al. (46). No mice were stopped because they refused to run further due to exhaustion. Therefore, in this EHS model, the physiologic end point was “symptom limited” by central nervous system dysfunction.

Typical thermal profiles at each temperature are shown in Fig. 1. During preincubation, before the beginning of the exercise protocol, the mice that were exposed to 37.5°C at 50% RH (Fig. 1A) allowed \( T_{c} \) to rise from the normal daytime baseline of 36–36.5°C to approach the \( T_{env} \) of 37.5°C. Similarly, animals exposed to 38.5 or 39.5°C (Fig. 1, B or C) allowed resting \( T_{c} \) to elevate to within 0.5°C of \( T_{env} \). Therefore at 50% RH, the animals started exercising at different \( T_{c} \) values, initially maintaining only very small gradients with \( T_{env} \). One interpretation of this is that the capacity (or willingness) of mice to remove metabolic heat within this range is extremely limited and they seem to be programmed to thermoregulate only enough to maintain \( \approx 0.5°C \) gradient with the \( T_{env} \). Interestingly, at the beginning of the exercise protocol, at each \( T_{env} \), there was an immediate elevation in \( T_{c} \), bringing all animals to approximately the same \( T_{c} \) of \( \approx 40°C \), independent of \( T_{env} \). This means that throughout the exercise period all mice were in a state in which their \( T_{c} \) had risen high enough to produce an initial net gradient of 0.5–2.5°C from \( T_{c} \) to \( T_{env} \), allowing for some radiant heat loss. During exercise, the initial metabolic heat production in each \( T_{env} \) condition was approximately the same, because each animal was put through the same initial incremental exercise protocol. Based on the \( T_{c} \) profiles, it appears that the animals exposed to \( T_{env} \) of 37.5 or 38.5°C initially made reasonable attempts at defending a \( T_{c} \) of \( \approx 40°C \) in the face of increasing metabolic heat production until it became too great to dissipate. At that point \( T_{c} \) began to rapidly rise, leading to heat stroke symptoms. In contrast, at 39.5°C the rise in \( T_{c} \) was almost linear with the initiation of exercise, suggesting that these animals simply could not disperse the excess heat from the metabolic load against the small gradient between \( T_{c} \) and \( T_{env} \).
Near Tc,max, there was a rapid rise in Tc, shortly before neurological symptoms. Tc,max was often reached shortly after neurological symptoms became limiting and the running protocol was stopped. After reaching the end point of the EHS protocol, the door of the environmental chamber was kept open, the animals returned to their cages, and the set point of the chamber lowered to 25°C (room temperature) and 50% RH. During this period a rapid fall in Tc into a hypothermic range was observed during the first h of recovery. These changes are qualitatively similar to PHS model in unanesthetized mice (46). We could not detect a secondary fever during recovery after the hypothermic period that was previously shown in PHS (46), although we only followed these animals over a 24-h recovery (Fig. 1, A–D, insets). In EXC animals, there was a reproducible elevation in Tc of 2°C during the exercise protocol to an average maximum of 38.4 ± 0.1 (SD) °C (Fig. 1F).

The Tc,max values achieved in each experimental group are shown in Fig. 2A. These were not statistically different between groups studied at the different levels of Tenv, while in 50% RH, the average ranging from 42.1–42.5°C. However, the exercise time required to attain symptom-limited EHS was significantly different between groups (Fig. 2B), requiring only ~61 min at 39.5°C at 50% RH but ~113 min at 37.5°C at 50% RH. In addition, both the distance run in meters, and the maximum running speed attained during the exercise protocol were much greater at the lower temperatures (Table 1). Thermal areas (Fig. 2C), i.e., the integration of temperature over time (°C·min), as described by Leon et al. (46), were significantly elevated as the level of Tenv was decreased. The highest thermal area was in the 37.5°C group (144 ± 21°C·min) but was still substantially lower than values observed in models of PHS in awake mice exposed to 39.5°C, i.e., 275–356°C·min (46). All animals lost between 6–8% of body weight during the test (Fig. 2D), and there were no significant differences between groups. Other major outcome variables are summarized in Table 1. Briefly, there were no differences in baseline measurements between groups. The average minimal core temperature attained over 30 s (Tc,min) and the profiles during the hypothermic phase of recovery were not significantly altered by the different Tenv exposures at 50% RH exposure.

**Effects of environmental humidity.** In general, the responses at 37.5°C at 30% RH resembled the responses of 37.5°C at 50% RH and were not significantly different for any variable. However, the responses to 37.5°C at 90% RH were unexpectedly altered. The mice reached symptom-limited Tc,max at an average of 41.5 ± 0.2°C, significantly lower than observations at both 30 and 50% RH (Fig. 3A). Surprisingly, the 37.5°C at 90% RH group were able to do this while running for longer distances (Fig. 3B) at faster running speeds (Table 2). Of note, the 90% RH group had a similar total thermal area (Fig. 3C) and percent weight loss compared with the other two RH groups (Fig. 3D). However, hypothermia depth was significantly shallower, possibly corresponding with the lower Tc,max reached. Subjectively, the fur of all the mice exposed to 90% RH (but not 30 or 50% RH) was saturated with moisture at the end of the exercise period.

The temperature profile of the 90% RH mice had other attributes that were different from the other two RH groups (Fig. 1, A and D). The 90% RH animals showed a rapid rise in Tc during the preincubation period, again reaching a Tc of ~40°C before the initiation of exercise, very similar to the animals exposed to 39.5°C (Fig. 1C). This means that at high humidity the animals were willing to allow Tc to elevate...

Fig. 1. A–F: thermoregulatory profiles at varying temperature and humidities. Insets: slower time scales to demonstrate late thermoregulatory responses during recovery. Broken lines: time of beginning the elevation in temperature in the environmental chamber. Dashed line: beginning of exercise.
again to this common 40°C threshold, but providing them a 2.5°C gradient to dissipate heat, but with a limited ability to dissipate it through evaporative processes. The striking difference in response is that once they achieved this 40°C set point they were able to defend it for an extended period, far exceeding the duration of the EHS protocols in all other conditions. We suggest that this reflects the ability to dissipate heat by radiation across the wet fur.

Histological evidence for injury to liver, kidney, and intestine. To evaluate organ injury in this model, random samples of liver, kidney, and small intestine were obtained at time points where corresponding damage biomarkers were most evident (discussed below). These measurements were made in the animals exposed to 37.5°C and 50% RH. The liver, kidney, and small intestine were selected because they have been known to be highly susceptible to heat injury (62). Kidney damage was measured at the 0.5-h recovery time point (Fig. 4, A and B). The tissues from EHS mice showed visible vascular congestion, hemorrhage, and thrombi. Typical liver tissue histopathology, measured at 24 h post-EHS, is shown in Fig. 4, C and D. These images also suggest hemorrhage, vascular congestion, and apparent thrombus formation in large vessels. None of these changes were apparent in EXC controls. We analyzed intestinal injury at 0.5 h into recovery, based on evidence of injury in previous studies at this time point using PHS models (52, 54). As shown by examples in Fig. 5, visible evidence of intestinal injury was prominent in all three segments of the small intestine. The results of quantitative analyses from all animals are shown in Fig. 6, A–C. There were consistently higher levels of injury in all three areas of the small intestine in EHS mice compared with EXC mice (Fig. 6A). Villus length, used as a measure of the process of restitution, usually appears quickly after villus injury (18). At the 0.5-h time point there were no significant differences in villus length between EXC and EHS (Fig. 6B), which is in contrast to other models of PHS in anesthetized mice (52, 54).

Table 1. Effects of environmental temperatures on EHS

<table>
<thead>
<tr>
<th>T&lt;sub&gt;e&lt;/sub&gt; Response</th>
<th>39.5°C</th>
<th>38.5°C</th>
<th>37.5°C</th>
<th>P Value (ANOVA)</th>
<th>PHS 39.5/50%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial weight, g</td>
<td>30.7±1.6</td>
<td>29.4±2.5</td>
<td>30.7±2.4</td>
<td>0.500</td>
<td>28.1±1.1</td>
</tr>
<tr>
<td>Time to reach T&lt;sub&gt;e&lt;/sub&gt; minimum, min</td>
<td>178.8±26</td>
<td>204.6±24†</td>
<td>250.9±29.7‡</td>
<td>0.001*</td>
<td>483±64</td>
</tr>
<tr>
<td>T&lt;sub&gt;e&lt;/sub&gt; minimum temp, °C</td>
<td>32.8±0.9</td>
<td>32.2±1.3</td>
<td>32.6±1.2</td>
<td>0.112</td>
<td>28.7±1.0</td>
</tr>
<tr>
<td>Distance, m</td>
<td>196.2±39†</td>
<td>316.0±32.5†</td>
<td>444.9±89.3‡</td>
<td>0.0001*</td>
<td>n/a</td>
</tr>
<tr>
<td>Hypothermia depth, °C</td>
<td>33.2±0.9</td>
<td>32.6±1.4</td>
<td>33.0±1.1</td>
<td>0.679</td>
<td>29.2±1.1</td>
</tr>
<tr>
<td>Hypothermia length, min</td>
<td>141.2±75.9</td>
<td>199.6±100</td>
<td>193.8±51.8</td>
<td>0.387</td>
<td>n/a</td>
</tr>
<tr>
<td>Hypothermia transition time, min</td>
<td>74.8±53.7</td>
<td>127.1±77.1</td>
<td>99.2±56.2</td>
<td>0.382</td>
<td>n/a</td>
</tr>
<tr>
<td>Ascending thermal area, °C · min</td>
<td>48.9±13.7</td>
<td>69.7±16.4†</td>
<td>96.5±14.7‡</td>
<td>0.0002*</td>
<td>223±90</td>
</tr>
<tr>
<td>Descending thermal area, °C · min</td>
<td>9.5±2.5</td>
<td>6.7±2.1</td>
<td>6.1±3.0</td>
<td>0.079</td>
<td>12.4±3.7</td>
</tr>
<tr>
<td>Maximum speed, m/min</td>
<td>3.7±0.4</td>
<td>4.6±0.15†</td>
<td>5.3±0.6‡</td>
<td>0.001*</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 6 in each group. ANOVA is for main effect of environmental temperatures (T<sub>env</sub>). Core temperature (T<sub>e</sub>) minimum is the lowest average value attained over 30 s and hypothermia depth is the lowest 1-h average T<sub>e</sub> during recovery. EHS, exertional heat stroke; PHS, passive heat stroke. *P < 0.05, significantly different. †Difference from 39.5°C, ‡difference from 38.5°C by post hoc comparisons.
Across all three regions, there were small but significant elevations in villus width by main effects in ANOVA ($P < 0.02$; Fig. 6C) but within individual areas of the intestine, this did not reach statistical significance by post hoc analyses. Villus width is generally indicative of acute swelling and vascular congestion (69).

**Biomarkers for organ damage.** A variety of common plasma biomarkers were used to detect damage to specific organ systems over the course of recovery from EHS. Biomarkers were chosen because of their ability to quantify estimates of specific injury over time and because of the potential for translation to clinical applications. ALT, a marker of liver injury, was significantly elevated to the same extent at 0.5 h in both EHS and EXC (Fig. 7A). However, by 3 h, while EXC levels returned to normal, ALT continued to rise, reaching median peak value of $>100$ U/l at 24 h. The 95% confidence interval (CI) for ALT in a large population of adult C57Bl/6 mice was 17–25 U/l (22), a range very similar to our NC group.

Therefore, the elevation in ALT is consistent with significant liver injury. In most animals, ALT was still elevated at 4 days [mean value $= 132 \pm 153$ (SD) U/l], but this did not reach statistical significance compared with NC mice.

CK was used as a marker of skeletal and/or cardiac muscle damage and displayed a unique time course (Fig. 7B). Damage was not evident at 0.5 h of recovery but was significantly increased at 3 h compared with EXC and NC. Surprisingly, it then returned to values similar to baseline or NC at 24 h, followed by a marked late-phase response at 4 days (96 h) that was significantly elevated compared both EXC and NC.

Kidney function was evaluated using BUN (Fig. 7C) and its relationship to creatinine (Fig. 7D). Elevations in BUN were markedly higher than both EXC and NC at 0.5 h post-EHS and were still higher in EHS compared with NC at 3 h. However, by this time point, these values generally fell within what is considered the normal range for mice (22). Interestingly, at 24 h, we observed a significantly lower median BUN value (17 mg/dl) compared with both EXC and NC mice. Elevations in BUN can indicate acute renal failure, increased amino acid catabolism, dehydration, and/or gastrointestinal bleeding, while reductions in BUN are typical of liver failure (23). Since all of these conditions can be present in heat stroke, we attempted to further clarify the interpretation of BUN by measuring creatinine and calculating the BUN/creatinine ratio (Fig. 4D). Creatinine levels were never significantly elevated.

### Table 2. Effects of environmental humidity on EHS

<table>
<thead>
<tr>
<th>$T_c$ Response</th>
<th>37.5/90%</th>
<th>37.5/50%</th>
<th>37.5/30%</th>
<th>$P$ Value (ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial weight, g</td>
<td>$30.0 \pm 0.6$</td>
<td>$30.7 \pm 2.4$</td>
<td>$28.8 \pm 0.6$</td>
<td>0.309</td>
</tr>
<tr>
<td>Time to reach $T_c$, minimum, min</td>
<td>$242.5 \pm 22.8$</td>
<td>$250.9 \pm 29.7$</td>
<td>$246.1 \pm 16.8$</td>
<td>0.755</td>
</tr>
<tr>
<td>$T_c$, minimum temp, °C</td>
<td>$33.6 \pm 0.40$</td>
<td>$32.6 \pm 1.2$</td>
<td>$31.8 \pm 0.4$</td>
<td>0.067</td>
</tr>
<tr>
<td>Distance, m</td>
<td>$802.0 \pm 62.7$</td>
<td>$444.9 \pm 89.3$</td>
<td>$503.6 \pm 43.7$</td>
<td>0.008*</td>
</tr>
<tr>
<td>Hypothermia depth, °C</td>
<td>$34.2 \pm 0.4$</td>
<td>$33.0 \pm 1.1$</td>
<td>$32.2 \pm 0.4$</td>
<td>0.039*</td>
</tr>
<tr>
<td>Hypothermia length, min</td>
<td>$83.8 \pm 38.6$</td>
<td>$193.8 \pm 51.8$</td>
<td>$231.0 \pm 25.0$</td>
<td>0.035*</td>
</tr>
<tr>
<td>Hypothermia transition time, min</td>
<td>$27.2 \pm 13.0$</td>
<td>$99.2 \pm 56.2$</td>
<td>$147.6 \pm 18.5$</td>
<td>0.007*</td>
</tr>
<tr>
<td>Ascending thermal area, °C · min</td>
<td>$161.9 \pm 8.5$</td>
<td>$96.5 \pm 14.7$</td>
<td>$137.0 \pm 29.8$</td>
<td>0.009*</td>
</tr>
<tr>
<td>Descending thermal area, °C · min</td>
<td>$4.8 \pm 0.5$</td>
<td>$6.2 \pm 3.0$</td>
<td>$9.5 \pm 1.1$</td>
<td>0.033*</td>
</tr>
<tr>
<td>Maximum speed, m/min</td>
<td>$7.1 \pm 0.6$</td>
<td>$5.2 \pm 0.6$</td>
<td>$5.4 \pm 0.2$</td>
<td>0.006*</td>
</tr>
</tbody>
</table>

Values are means ± SD; $n = 6$ in each group. ANOVA $P$ values are main effect of relative humidity. *$P < 0.05$, significantly different. †Difference from 90% RH, ‡difference from 50% by post hoc comparisons.
post EHS, but at 3 h, the median level for plasma creatinine was 0.1 mg/dl, significantly lower than NC ($P < 0.002$) and EXC ($P < 0.05$) (data not shown). Creatinine was similar in all groups at 0.5 and 24 h and 4 days. The 95% CI for creatinine in mice is 0.49–0.55 mg/dl (22). The BUN/creatinine ratios (Fig. 4D) were significantly higher at 0.5 h compared with NC for both EHS and EXC groups, but in EHS the ratio continued to rise, generally reaching peak values at 3 h. High BUN/creatinine ratios can indicate acute renal failure but are also elevated in shock, severe dehydration, and/or gastrointestinal bleeding (23). At 24 h into recovery, BUN/creatinine dropped below NC levels ($P < 0.005$). A low ratio can indicate low levels of urea formation from reduced protein intake, liver disease, and/or rhabdomyolysis (23), all likely to be present and therefore not particularly specific for renal injury.

Intestinal damage was suggested by elevations in the intestinal FABP2 plasma biomarker. FABP2 is a protein found in enterocytes of the small intestinal epithelium. Upon cellular damage, it is released into the circulation (1). Peak FABP2 concentration after 0.5 h of recovery was significantly higher in EHS than both NC and EXC (Fig. 8). EXC animals also showed levels higher than NC at 0.5, 3, and 24 h. Although EHS concentrations of FABP2 decreased considerably over time, they were still significantly higher than NC at 3 and 24 h. Interestingly, FABP2 was higher in EXC than EHS at 24 h.

**Comparisons with PHS.** Extensive work had been done in PHS models in the mouse by Leon and colleagues (45, 46) in terms of organ damage and cytokine secretion. However, the mildest level of PHS in those studies had an average $T_{c,max}$ end point of 42.4°C, which was 0.3°C higher than our average peak $T_{c,max}$ for the groups studied for tissue injury (i.e., 37.5°C at −50% RH). For this reason we wished to evaluate the types of injury that would occur at this lower $T_{c,max}$ in PHS to compare directly with our EHS model. We followed the animals only to the 3-h recovery, a point in time where plasma biomarkers of injury are most often near maximum. The mice in the PHS group ($n = 6$) had an average thermal area of 409 ± 71 (SD) ($°$C·min) compared with 144 ± 22 (SD) in the EHS group ($°$C·min). Therefore, though the two groups reached the same $T_{c,max}$, the animals in the PHS group were exposed to over two times the heat dose. As shown in Fig. 7, the median ALT, BUN, and BUN/creatinine either met or exceeded the levels seen in EHS at the 3-h time point (none of which were significantly different from EHS). However, there was no evidence for elevations in CK at this time point, suggesting that muscle damage is not part of the constellation of problems in PHS, at least at 3 h, and at this $T_{c,max}$. Intestinal injury was also apparent, based on the elevations in FABP2 (Fig. 6), being significantly greater than both EHS and EXC at the same point in time ($P < 0.01$). Mean blood glucose was 64 ± 31 (SD) mg/dl in the PHS group, which was lower at this 3-h time point ($P < 0.05$) compared with both EXC and EHS groups and consistent with previous studies in the unanesthetized PHS mouse model (45).

**Blood glucose.** Nonfasted blood glucose levels were taken at the time of death, post-EHS or -EXC. As shown in Fig. 9, at 0.5 h into recovery, blood glucose was significantly lower in EHS compared with EXC or NC, reaching average levels of 47 ± 14 mg/dl compared with EXC [250 ± 46 (SD) mg/dl] or NC [110 ± 30 (SD) mg/dl]. No differences in glucose were noted between EHS, EXC or NC at 3 h (Fig. 9B). However, after 24 h of recovery, in both EHS and EXC, blood glucose values were significantly higher than NC. For the 4-day measurements (Fig. 9D), the glucose tests were only available in
the EHS groups receiving 30% and 90% RH at 37.5°C. However, similar results were seen in both groups with the extreme levels of RH. Mean glucose in these groups combined was 342 ± 68 mg/dl, a nonfasting value and range that is considerably higher than could be expected from either the effects of handling stress and/or isoflurane anesthesia. Both of these possible confounders independently elevate plasma glucose in mice (7, 15).

**DISCUSSION**

We have identified a unique preclinical model of EHS that closely resembles the pattern or response observed in humans. A primary outcome that distinguishes this approach is that the end point is determined by neurological symptoms, i.e., transient loss of motor control and consciousness. These characteristics are closely aligned with the clinical definitions of heat stroke (3) and what is generally observed in humans in EHS (62). Another feature is that EHS appears to induce organ system damage at a lower thermal “dose” as estimated by the thermal area. At a cellular or organ system level, the principles of tissue damage as a function of thermal “dose” (temperature and time of exposure) have been well established (20, 71) and have been applied to conditions of heat stroke in animal models by Hubbard et. al. (33) and Leon et al. (46). Extensive evidence of organ injury was observed in this model of EHS at a thermal dose of less than half of that created by the PHS protocol, when animals are brought to the same average Tc, max. This suggests that other pathological processes such as tissue ischemia,
increased metabolic load, or a more rapidly ascending temperature profile may accelerate the injury seen in EHS compared with PHS. In addition, when thermal load is superimposed on metabolic load, both of which induce their own patterns of gene expression or activation of cell signaling pathways, it would be expected to alter the pattern of responses compared with either thermal stress or exercise alone. Another unique feature, consistent with human EHS, is the presence of extensive rhabdomyolysis, which was not present in a matched model of PHS (Fig. 7B). Since rhabdomyolysis is a contributor to underlying secondary pathologies, such as kidney failure and disseminating intravascular coagulation, this may be a critically important aspect of the unique integrative responses to EHS.

Origins of multiple organ injury. Several different mechanisms are likely to converge to promote neurological dysfunction or unconsciousness during EHS, including reductions in cerebral blood flow from combined requirements of exercising muscles and heat exchange, dehydration, ongoing hypocapnia (24, 51), loss of blood-brain-barrier integrity (41), direct neurological effects of hyperthermia (67) and hypoglycemia. Of these mechanisms, the only variable we evaluated in this study was hypoglycemia, which was observed at 0.5 h of recovery (47 ± 14 mg/dl). This is within a range of values generally
considered “moderate” hypoglycemia in humans but at a level associated with significant cognitive impairment (2a). What this level of hypoglycemia means in a mouse is less clear. Normal values for blood glucose in mice can have a wide range. Some reported normal values are inordinately high when samples are taken in the awake state, e.g., 203–271, 95% CI (22), but the elevations have been shown to be proportional to the extent of handling of the mice and its impact on stress and cortisol levels (7). In chronically cannulated awake mice, normal nonfasting values still have a wide range but are much lower, i.e., 155 ± 28 (SD) mg/dl; these rise to 196 ± 76 in the same animals when put under mild isoflurane anesthesia (15). Our values for EXC and for NC mice, under baseline conditions and anesthetized with isoflurane, are within the 95% CI for this range. In PHS, Leon et al. (45) observed glucose values of 56 ± 16 mg/dl, ~3 h into recovery, but the average value at Tc,max was reduced by ~13 mg/dl. This suggests that it is likely that during the end stages of EHS, at the time of neurological symptoms, hypoglycemia in our EHS mice may have been more severe. The low delivery of glucose for normal nervous system function may have contributed to neurological symptoms, along with the other factors mentioned above.

The hyperglycemia that emerged late in the recovery process was an unexpected finding. The fact that it remained unresolved after 4 days of recovery suggests that it may reflect an underlying metabolic disorder, possibly due to liver, intestinal, muscle, or pancreatic injury, all of which provide hormonal signals or processes that directly control normal blood glucose. The pancreas is susceptible to ischemia in heat stroke due to reductions in splanchnic blood flow (29) and in animal models exposed to extreme hyperthermia (1 h at 45°C) the pancreas exhibits extensive histopathological damage (63). However, to our knowledge, pancreatic injury has not been carefully evaluated in documented heat stroke in humans or in models of survivable heat stroke. Exercise in the heat has been shown to increase the rate of glucose utilization (60), and this could put additional strains on pancreatic metabolism and on other organ systems involved with normal glucose homeostasis (liver and intestine). Furthermore, in exercise in the heat, as the splanchnic region is hypoxic, the resulting increases in lactate concentration in the liver can induce hyperglycemia (60). Whether

Fig. 8. Measurements of intestinal fatty acid binding protein-2 (FABP2) following exertional heat stroke: Data represent medians ± 25–75% quartiles (table below). See text for details. *P < 0.05 EHS vs. NC, #P < 0.05, EHS vs. EXC, ≥P < 0.05 EXC vs. NC; n = 6–7 per group.

Fig. 9. Blood glucose levels post-EHS. A: glucose, 0.5 h post-EHS or -EXC. B: 3 h post-EHS. C: 24 h post-EHS. D: 4 days post-EHS. See text for details. Data are medians ± 25–75% ranges. *P < 0.05, **P < 0.01.
this would be extended beyond the early recovery period would depend on what systems were damaged. Clinically, the hyperglycemia we observed after EHS is of interest, because in humans, heat stroke frequently presents with hyperglycemia at the time of admission (17, 31), which has proven to be among the best predictors of long-term heat stroke mortality (17). The hyperglycemia could also reflect artifacts of other underlying processes such as inflammation and stress. When a host responds to a pathogen there is an initiation of the inflammatory response resulting in background hyperglycemia (2). In addition, both catecholamines and corticosteroids are generally increased transiently over the first 24 h in heat stroke (28, 45), which can theoretically contribute to profound hyperglycemia. However, after 4 days, there is little evidence that corticosteroids or catecholamines would still be elevated (though this has not been studied extensively). Alternatively, it is also possible that following a traumatic event like EHS, mice are in a “vulnerable” psychological state that makes them extremely sensitive and more susceptible to the psychological stress from successive handling or the process of induction of anesthesia. However, based on measured effects of handling stress and/or isoﬂurane anesthesia on glucose and corticosteroids in the blood of control mice, the values we observed at 4 days are much higher than expected, suggesting an underlying loss of glucose homeostasis (7, 15).

Although we demonstrate that multiple organ damage was present by histological measurements in liver, kidney, and intestine at brief time windows of recovery, we depended on plasma biomarkers to evaluate the time course of ongoing injury. It is important to recognize that these measurements need to be viewed with caution, because elevations can reflect multiple ongoing processes that may or may not be a result of organ injury per se. The accumulation of biomarker proteins in the plasma is a net result of both production and clearance, so if clearance is reduced, such as might occur with kidney or liver damage, there may be an accumulation that is disproportionate to the extent or timing of injury.

Striated muscle injury was also a unique feature of this model compared with PHS and is consistent with the constellation of disorders typical of EHS in humans. Although elevations in CK are also evident in cardiac injury, in general, CK is still the most commonly used clinical indicator of skeletal muscle rhabdomyolysis (36). The time resolution and the interpretation of accumulated plasma CK measures are influenced by the long half-life, being 15 h for muscle isoforms in humans (43) but only ~1.5 h in mice (65). This may partially explain the somewhat unexpected time course of the response observed in mice, which demonstrated rather sharp increases and decreases in magnitude over time. No significant elevation was seen at 0.5 h, but at 3 h the accumulation in plasma was significant. By 24 h the elevation was greatly attenuated, but then the signal reappeared at 4 days (Fig. 7B). The later rise in CK at 4 days has some similarity to the delayed responses in CK seen after eccentric exercise (38) and may reflect some form of delayed muscle inflammation. Therefore, this late response raises the question as to whether some aspect of the type of exercise being performed in forced running wheels may have had a unique impact on CK release. Anecdotally, we have observed the behavior of the mice running on the forced running wheel to be quite different from behaviors on the treadmill. The running behavior includes considerable stretching and grasping of the spoked wheel, which could result in some eccentric motor movements, making delayed muscle injury possible. We did see very modest but significant elevations in CK in the EXC group performing exactly the same exercise but without hyperthermia.

The liver is a particularly vulnerable organ in hyperthermia. Recent models estimating temperature distributions in body compartments during heat stroke in rats have predicted that liver temperatures may be as high as 2°C above Tc (58). Previous studies by Hubbard et al. (34) also have suggested that liver enzymes may be among the most sensitive markers of heat injury, and it is thought to be the result of both hypoxia and direct heat damage (39). These markers are also commonly elevated in exhaustive exercise. For example, Hubbard et al. (34) demonstrated that 24 h after rats were run to exhaustion, elevations in ALT were modestly increased, even when exercising in the cold (5°C) but rose as a proportion to the Tenv during the run. We also saw small elevations in ALT at 0.5 h in EXC animals but the response quickly resolved to baseline by 3 h (Fig. 4A). Hubbard et al. (34) also observed that when rats are passively heated to 41.5°C the increase in ALT exceeded what was seen in exercise in the heat suggesting a complex relationship between exercise and hyperthermia in producing a given degree of liver damage. In contrast to Hubbard et al., we continued to see ALT elevations suggesting ongoing liver damage extending up to 4 days in three of the six mice studied. It has been hypothesized that even though liver damage generally peaks about 24 to 48 h after heat stroke, it may take much longer (i.e., weeks or months) to entirely repair this damage (9, 44, 50, 61). The interpretation of the timing of the liver injury as reflected by ALT measurements is complicated by the long half-life in plasma, being 47 h in normal humans (40). The half-life is less quantified in mice but decay following chemically induced liver injury suggests it is much less than 24 h (70).

Renal failure is commonly present in heat stroke patients, and BUN accumulation can be an indication of kidney damage or necrosis (50). Unfortunately, BUN, creatinine, and BUN/creatinine ratios are not particularly specific for renal injury or failure even though these are among the most common clinical indicators used (23). We were surprised to find that creatinine values were essentially equal to normal controls, with the exception of the 3-h time point, where they were significantly lower than both NC and EXC. Although plasma creatinine is commonly used to indicate acute renal injury in steady-state clinical conditions such as hypertension in humans (48), it is not recommended as an indicator for critically ill patients where hydration and organ function are not stable (49). In sepsis, which has many parallels to the pathophysiology of heat stroke, creatinine production from muscle is markedly reduced, making it of limited use in examining acute kidney injury (21). Furthermore, in dogs with fatal heat stroke, creatinine concentrations have been found to be normal at the time of admission, even with existing evidence of pathological renal lesions (13). It is of note that previous studies have also questioned the reliability of the creatinine values using common autoanalyzer systems, whereas BUN values have been validated (26). For this reason we relied on BUN measurements as our primary biomarker for renal function in this setting with the understanding of the extensive limitations to its interpretation.
The intestinal barrier has long been considered a major target for injury during heat stroke (12, 25, 30). Exertional heat stroke may be particularly susceptible to intestinal injury since in humans (68) and in mice (27); heavy endurance exercise alone can result in evidence for loss of the intestinal barrier function and enterocyte injury. The effects of intestinal injury can have important consequences for the progression of heat stroke. It can lead to the release of endotoxin or other inflammatory mediators into the blood, which can drive the immune system to initiate a systemic inflammatory response syndrome (SIRS), believed to contribute to organ injury and mortality in severe heat stroke (12, 45). Although the liver is usually responsible for the clearance of endotoxin, as the exercise intensity and/or the heat load increase, less blood flow to the liver and liver injury can potentially compromise this function (62). In our previous work in a PHS model in anesthetized mice, we found marked elevations in intestinal permeability that were more or less isolated to the duodenum (52, 53). In contrast, in this EHS model, damage is clearly present in all three segments of the intestine in essentially every sample we observed. The other marker of small intestinal damage we examined was FABP2. Intestinal enterocytes express FABP2 during early damage of the epithelial cell and the protein is released rapidly into the circulation (19). In PHS the level of FABP2 was extremely high, suggesting extensive injury in the unanesthetized PHS conditions as well.

Another difference we observed between the histological results we saw in this experiment compared with our previous analyses in anesthetized PHS models is that at this 0.5-h recovery window, there was no evidence of shortening of the intestinal villi, i.e., restitution. Restitution, or sloughing off of the tips of the damaged villi, is one of the chief mechanisms in the early stages of intestinal repair. In all of our previous analyses, this process was nearly complete within this time window. One possibility is that PHS protocols require 1–2 h of greater heat exposure to reach $T_{\text{max}}$. Therefore, the restitution process may have not had time to develop in this EHS model. Secondly, the injury may be so extensive in EHS that the epithelial cells lining the intestinal wall may not have recovered sufficiently to begin the repair process effectively.

Significance of the mouse model development. This is the first model of EHS in the mouse that resembles human EHS. It was developed to provide a preclinical instrument to evaluate treatment and prevention paradigms for EHS in humans, which remains a serious and unpredictable medical condition. Improvements in preventative and post-EHS care, particularly rapid cooling and appropriate diagnosis and monitoring, no doubt have raised the likelihood of survival in humans in recent years (6, 10). However, patients who survive may have long-term medical complications and susceptibilities to other causes of mortality that are still poorly understood (64). Therefore, improving survival rates may not be enough. New approaches to prevention, prediction of susceptibility, and development of new forms of treatment are greatly needed. Numerous questions remain unanswered such as why specific individuals succumb to EHS on specific occasions, comorbidities that are not currently recognized, the involvement of the immune system in both organ dysfunction and repair, and the influence of exercise conditioning and acute exercise on the evolution of the organ injury (62). These are all easiest to address in animal models, before human studies. Furthermore, there are technical advantages to having a mouse model, including the availability of mouse-specific assays and the ability to rapidly test mechanistic questions through genetically altered strains. The use of mice, rather than rats or other species, may also have been fortuitous, because once trained, mice will run willingly in the heat until they lose consciousness. This may reflect the fact that mice have a greater tendency for flight in response to threats (such as severe heat exposure), compared with the domesticated laboratory rat, which shows a greater tendency to freeze in response to threatening environments (11). This also suggests that even though we went to great lengths to avoid the use of electrical shock or startle stimuli to induce continued running, the mice probably experienced a very stressful state, and they responded by running to escape continued exposure. However, these natural stresses that were placed on the mice may be analogous to conditions humans often experience in EHS, where they push themselves beyond their normal capabilities due to competitive drive, coupled with psychological stressors inherent to combat or competition. Finally, the predictability of the mouse model across a variety of temperatures and humidities and the 100% survival rates in the face of relatively severe organ injury and dysfunction will facilitate identification of comorbidities and risk factors in future studies.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


