Heat stroke activates a stress-induced cytokine response in skeletal muscle

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Heat stroke (HS) induces a rapid elevation in a number of circulating cytokines. This is often attributed to the stimulatory effects of endotoxin, released from damaged intestine, on immune cells. However, parenchymal cells also produce cytokines, and skeletal muscle, comprising a large proportion of body mass, is thought to participate. We tested the hypothesis that skeletal muscle exhibits a cytokine response to HS that parallels the systemic response in conscious mice heated to a core temperature of 42.4°C (TcMax). Diaphragm and hindlimb muscles showed a rapid rise in interleukin-6 (IL-6) and interleukin-10 (IL-10) mRNA and transient inhibition of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) throughout early recovery, a pattern that parallels changes in circulating cytokines. IL-6 protein was transiently elevated in both muscles at ~32 min after reaching TcMax. Other responses observed included an upregulation of toll-like receptor-4 (TLR-4) and heat shock protein-72 (HSP-72) mRNA but no change in TLR-2 or HSP25 mRNA. Furthermore, c-jun and c-fos mRNA increased. Together, c-jun/c-fos form the activator protein-1 (AP-1) transcription factor, critical for stress-induced regulation of IL-6. Interestingly, a second “late-phase” (24 h) cytokine response, with increases in IL-6, IL-10, IL-1β, and TNF-α protein, were observed in hindlimb but not diaphragm muscle. These results demonstrate that skeletal muscle responds to HS with a distinct “stress-induced immune response,” characterized by an early upregulation of IL-6, IL-10, and TLR-4 and suppression of IL-1β and TNF-α mRNA, a pattern discrete from classic innate immune cytokine responses.

IL-6; IL-10; heat stress; myokines; toll-like receptors

Skeletal muscles are now recognized as an endocrine source of circulating IL-6 and other cytokines during exercise (50, 61, 69) and may respond similarly to other forms of stress. For example, we have shown that skeletal muscle myotubes secrete abundant IL-6 in response to hyperthermia, protein damage, or activation of the unfolded protein response (66, 67). This raises the possibility that skeletal muscles might play a role in the integrated stress response of organisms to heat exposure or heat stroke (HS), particularly since increased IL-6 is among the most profound cytokines responding in all models of HS. There are many possible signaling pathways that could account for stimulation of muscle cytokine production in conditions of HS (65). However, two candidates are likely to be involved. The first arises from the influence of pathogen- or damage-associated molecular patterns (PAMPs and DAMPs) such as endotoxin or high-mobility group box 1 (HMGB1), released during HS (10, 18). Skeletal muscles have been shown to be very capable of responding to these signals by activation of an abundance of toll-like receptor isoforms (TLRs) present on the muscle fibers (10, 16, 30). The second possibility is a direct influence of hyperthermia or secondary stress signaling that occurs in muscle fibers during heat exposure or during conditions of protein stress (66, 67).

The goal of this study was therefore to quantify the acute gene expression profiles for cytokines and other immune response proteins expressed in skeletal muscle in response to HS (severe hyperthermia) and to compare these with the pattern of changes in cytokines seen in the circulation. We then used these data to distinguish between two competing hypotheses regarding the origins of acute cytokine signaling in muscle in these conditions. The first hypothesis was (H1) skeletal muscle gene expression reflects a direct response of muscle to PAMPs or DAMPs, released during heat exposure. If H1 were true, we would expect the pattern of skeletal muscle gene expression to resemble the direct response to endotoxin, previously documented in skeletal muscle (16, 30) or of exposure to DAMPs such as HMGB1, which would be expected to induce similar patterns of response (2, 15). The alternative hypothesis was (H2) skeletal muscle gene expression reflects a direct response to stress signaling due to heat exposure. If H2 were true, the pattern of cytokine gene expression would resemble the profile observed in isolated muscles or myotubes exposed to hyperthermia alone, in the absence of endotoxin (66, 67).

To address this problem, we utilized a physiologically relevant, conscious HS model in mice that has been validated with observations made in humans suffering from HS and for which the acute circulating cytokine responses have been characterized (19, 21, 32). We identified the temporal pattern of mRNA and protein changes of cytokines and the upstream signaling pathways in muscle that are involved with cytokine regulation. These latter measurements included TLRs, heat shock proteins.
(HSPs), and the early stress genes, c-jun and c-fos. We also compared these responses in different muscles, intermittently contracting (hindlimb) and constitutively contracting (diaphragm) muscles.

We demonstrate that the early responses of cytokines, TLRs, and stress signaling molecules within skeletal muscles are consistent with an “early phase” stress-induced cytokine response. This supports hypothesis H2 and also reflects changes seen in the circulating cytokine profile during early stages of recovery from HS. These patterns are distinctly different from the responses expected from an acute response of muscle to endotoxin or other TLR activators.

MATERIALS AND METHODS

Animals. Specific pathogen-free 3–5 mo old male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were used. Mice were individually housed in Nalgene polycarbonate cages (11.5 × 7.5 × 5 in) fitted with HEPA-filter cage tops and Alpha-Dri bedding (The Andersons, Maumee, OH). Rodent laboratory chow (LM-485; Harlan Teklad, Madison, WI) and water were provided ad libitum under standard laboratory conditions (25 ± 2°C, 12:12 h light-dark cycle, lights on at 0600) (32). In conducting research using animals, we adhered to the Guide for the Care and Use of Laboratory Animals (46) 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 and all experiments were carried out at the USARIEM (Natick, MA).

Tissue harvesting and blood collection. Tissues and blood were collected from C57BL/6J mice as described in detail previously (32). In brief, C57BL/6J mice were randomly assigned to the following groups for tissue collection (chronologically): sample collection at time 0 (TC, gene, and protein expression. C57BL/6J mice were rapidly anesthetized (<1 min) with isoflurane (5% in 100% O₂, flow rate 3.0 L/min) and exsanguinated following thoracotomy and intracardiac puncture (1 ml EDTA-coated syringe, 23-gauge needle). Blood was transferred to 1.5 ml EDTA-treated microcentrifuge tubes and immediately placed onto ice until plasma could be separated by centrifugation (4°C; 5 min, ~3,500 rpm). Plasma samples were aliquoted into 150 µl volumes and stored at −80°C until assayed. The diaphragm and hindlimb muscles were rapidly excised, frozen in liquid nitrogen, and stored at −80°C until assayed.

Tₐ measurements. Tₐ (≥0.1°C) was continuously monitored at 1-min intervals by radiotelemetry in conscious, freely moving mice using the VitalView Data Acquisition system (Mini Mitter, Bend, OR). 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 battery-free temperature-sensitive transmitter (1.1 g, model G2 Emitter) was implanted intra-abdominally using aseptic technique. Frequency of the emitted transmitter signal is proportional to Tₐ. An antenna placed under each animal’s cage received the emitted transmitter signal and converted it to Tₐ using predetermined calibration values. Analgesia was provided as a 190 mg Dustless Precision Pellet (Product #F6699, F6699, F6700, and F6701: Bio-Serv, Frenchtown, NJ) that contained 25 µg of indomethacin (Sigma #I-8280, St. Louis, MO). Pellets were placed onto the cage floor for voluntary consumption ~1 h prior to surgery and at 0800 h on days 1, 2, and 3 of recovery. Daily consumption of the pellet was visually confirmed. Surgical recovery (~14 days) was required before HS experimentation, as defined by a return to presurgical body weight and manifestation of a robust, consistent circadian Tₐ rhythm, as previously described for this species (36).

Heat stress and sampling protocol. The HS protocol has been described in detail elsewhere (33). Briefly, conscious, unrestrained mice were exposed to an ambient temperature (Tₐ) of 39.5 ± 0.2°C in an incubator, in the absence of food and water, until Tₐ of 42.4°C was attained. Following removal from the heat at Tₐ, food and water were provided ad libitum during undisturbed recovery at Tₐ of 25 ± 2°C. This relatively cool recovery Tₐ [below the thermoneutral zone for this species (17)] was chosen, since it was previously shown to support hyperthermia and fever development and is essential for HS recovery in this species (33).

The inclusion criteria and group designations have previously been described (32) (Table 1 and Fig. 1). Prior to HS, the animals maintained daytime Tₐ at 36.0°C (12 h average 0600—1759), which represents the normal Tₐ nadir in this species (33). The level of Tₐ (42.4°C) represents a mild HS associated with 100% survival in our model. RTB was the time point when TC descended back to 36°C following removal from the heat. HD was the lowest TC observed during the hypothermic period of recovery at TC of 25°C. It was previously determined that the depth of hyperthermia during recovery at this Tₐ is characterized by a cooling rate of 0.01°C/min (33); therefore, when this cooling rate was observed, samples were collected for the HD group. Last, tissues from the 24 h recovery group were harvested 24 h following the start of heat exposure.

Chemicals and reagents. RiboPure Kit (Ambion, Austin, TX), High Capacity Reverse Transcription Kit, Taqman Advanced Fast Master Mix (Applied Biosystems, Foster City, CA), Tissue Protein Extraction Reagent (T-PER), HALT protease and phosphatase inhibitor cocktail, BCA Protein Assay Kit (Thermo Scientific,; Rockford, IL), Mouse IL-6, IL-10, IL-1β, and TNF-α Platinum ELISA kits (eBioscience, San Diego, CA), and 10% neutral-buffered formalin (Carson Millonig Formulation, Fisher Scientific, Springfield, MA).

RNA isolation and reverse transcription. Muscles were placed in TRI-reagent and homogenized, and RNA was isolated according to the manufacturer’s instructions. Briefly, RNA was separated from protein and DNA by the addition of 1-Bromo-3-chloropropane and...
precipitation in isopropanol. After a 75% ethanol wash and resuspension in RNase-free H2O, purity of RNA samples was quantified using a Nanodrop 8000 spectrophotometer (Nanodrop Products, Wilmington, DE). RNA concentration was calculated from the 260 nm reading. The 260:280 nm ratios for all RNA samples used in RT-PCR analysis were ≥1.8. RNA samples were reverse transcribed into cDNA using the High Capacity Reverse Transcription Kit.

**Real-time PCR.** Preformulated TaqMan Gene Expression Assays were purchased from Applied Biosystems for the following mouse genes: Interleukin-6 (IL-6), Heat Shock Protein 1A (HSP72), HSP25, Beta Actin (ACTB), Hypoxanthine Guanine Phosphoribosyl Transferase (HPRT), IL-10, TLR-2, TLR-4, c-jun, c-fos, TNF-α, and IL-1β. Relative quantitative real time RT-PCR was performed using the TaqMan Fast Advanced Master Mix, and reactions were performed in duplicate on a StepOnePlus Real-Time PCR System (Life Technologies) according to the following protocol: hold at 95°C for 20 s, 40 cycles of 95°C for 1 s and 60°C for 20 s. Housekeeping gene candidates HPRT and ACTB were tested for stability over various experimental treatments as previously shown (67). HPRT was chosen as the housekeeping gene and used to normalize target gene expression. Changes in target gene expression were independent of changes in the level of mRNA for HPRT. Relative quantitation was calculated using the ΔΔCT method as described previously (21). Sample sizes range from six to eight mice per group.

**Protein extraction and quantification.** Muscle samples were placed in equal volumes of ice cold T-PER containing HALT protease and phosphatase inhibitor cocktail and ground into a fine pulp. The lysate was transferred to clean microcentrifuge tubes and stored at −80°C for subsequent analysis. Total protein was quantified using the BCA Protein Assay Kit.

**ELISA.** IL-6, IL-1β, IL-10, and TNF-α Platinum ELISAs were purchased from eBioscience. Frozen muscle homogenates were brought to room temperature and diluted to 0.5 mg/ml total protein. Samples of uniform total protein concentration were pipetted on a 96-well plate. Each sample was added to a well and incubated for 2 h at room temperature. A biotin-conjugated anti-mouse IL-6, IL-10, TNF-α, or IL-1β antibody was added to each well, followed by incubation for 1 h at room temperature. Streptavidin-Horseradish Peroxidase (HRP) was added to each well. Each well was then added to the wells to generate a colored product proportional to the amount of mouse (IL-6, IL-10, TNF-α, IL-1β) present and measured at 450 nm. Concentrations of samples in pg/ml were interpolated using a five-parameter logistic standard curve, using Prism Software 5.0 (Graph-Pad Software). Cytokine protein expression was quantified and normalized to total protein content and converted into arbitrary units for ease of interpretation. Sample sizes range from six to seven mice per group.

**Statistics.** Results of PCR data are presented as bar graphs of the mean fold change without error bars. This is necessary as cycle threshold is exponentially related to copy number, which is converted to fold change to derive linear comparisons. Results of cytokine protein data are presented as values of central tendency, expressed as means ± SE. In mouse HS experiments, data was analyzed using the Kruskal-Wallis one-way ANOVA because this test does not assume normal distribution and data sets were routinely not normally distributed. A minimum statistical significance was set at $P < 0.05$.

**RESULTS**

**Heat stress characteristics.** Figure 1 shows a typical Tc curve from one sham control and one HS mouse through 24 h of recovery at 25°C. The thermoregulatory profile for this model has previously been described in detail (33). Briefly, mice exhibited a triphasic hyperthermic response during heat exposure from baseline (time 0, Tc = 36°C) to attainment of TcMax. As summarized in Table 1, following removal from heat and placement into a 25°C environment, Tc returned to baseline Tc of 36°C (RTB), requiring an average of 32.4 min from TcMax. Then the mice developed a profound hypothermia, reaching a minimum Tc of 30.8 ± 0.8°C (HD), occurring at ~140.9 min after RTB. Later, 5–6 h after TcMax, Tc returned to baseline temperature. A fever-like Tc elevation was observed at ~24 h from the beginning of the protocol (17.7 h from HD).

**Muscle cytokine gene expression at peak HS and in the early recovery period.** IL-6, IL-10, IL-1β, and TNF-α gene expressions were evaluated in both the diaphragm and soleus in the early recovery period (Fig. 2). Sham control mice showed no

### Table 1. Change in time and core temperature in mice that underwent HS protocol

<table>
<thead>
<tr>
<th>Time, min</th>
<th>Tc (°C)</th>
<th>RTB (°C)</th>
<th>HD (°C)</th>
<th>24 h (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>36</td>
<td>206 ± 6.3</td>
<td>238.4 ± 0.9</td>
<td>379.3 ± 8.0</td>
</tr>
<tr>
<td>Δ time, min</td>
<td>--&gt;206</td>
<td>--&gt;32.4</td>
<td>--&gt;140.9</td>
<td>--&gt;0.8°C</td>
</tr>
<tr>
<td>Recovery phase</td>
<td>36°C</td>
<td>42.4°C</td>
<td>HS</td>
<td>early</td>
</tr>
</tbody>
</table>

Values are means ± SE. Conscious, unrestrained C57BL/6j mice individually housed were exposed to an ambient temperature (Tc) of 39.5 ± 0.2°C in an incubator until a maximum core temperature (TcMax) of 42.4°C was attained. Mice recovered at Tc of 25 ± 2°C. The average longitudinal timeline (Time) and the average change in time between recovery periods (ΔTime) are provided in minutes (min). Also included are the average Tc observed at each respective time point and classification of the recovery phase as control, HS, early, or late recovery as referred throughout the text. Sample size was 6-7 mice per group.
differences in IL-6, IL-10, IL-1β, or TNF-α gene expression at any time point within either muscle group. In HS animals, IL-6 mRNA expression was significantly increased in diaphragm muscle (Fig. 2A, dark) at the time points, RTB (21.6-fold, \( P < 0.01 \)) and HD (34.3-fold, \( P < 0.05 \)). In the soleus (Fig. 2A, white), IL-6 mRNA was also significantly increased at RTB (9.9-fold, \( P < 0.01 \)). The response was brief in the soleus. By HD, IL-6 mRNA was equivalent to control. Therefore, during the early recovery phase, IL-6 gene expression in the diaphragm was elevated for an extended period compared with the soleus.

Diaphragm IL-10 mRNA (Fig. 2B, dark) modestly decreased at \( T_{\text{Cmax}} \) compared with control (0.3-fold, \( P < 0.05 \)) and compared with HS soleus (\( P < 0.05 \)). However, IL-10 mRNA quickly peaked at RTB (15.8-fold, \( P < 0.01 \)) and remained elevated through HD (8.1-fold, \( P < 0.05 \)) in the diaphragm. In the soleus (Fig. 2B, white), IL-10 mRNA was significantly upregulated, transiently at RTB (10-fold, \( P < 0.01 \)), a response closely resembling the pattern of response for IL-6.

IL-1β (Fig. 2C, dark) and TNF-α (Fig. 2D, dark) mRNA expression did not significantly increase in the diaphragm at any time point compared with control muscles. However, diaphragm TNF-α mRNA was decreased compared with control (0.1-fold, \( P < 0.05 \)) and HS soleus (\( P < 0.01 \)) at \( T_{\text{Cmax}} \). In the soleus, IL-1β (Fig. 2C, white) and TNF-α (Fig. 2D, white) mRNA expression also did not increase significantly at any time point, but IL-1β mRNA was significantly decreased at \( T_{\text{Cmax}} \) (0.5-fold, \( P < 0.05 \)).

IL-6, IL-10, IL-1β, and TNF-α protein expression in skeletal muscle during early and late HS recovery. Protein measurements were used to determine if synthesized cytokine protein, stored within the muscle, followed the elevations seen in mRNA. The homogenates of the remaining contralateral soleus and another section of the costal diaphragm (matched to the mRNA measurements) were first used for muscle IL-6 protein determinations. Because of low protein yields from the soleus, only measurements of IL-6 could be evaluated at each time point, but comparisons were made from matched diaphragm tissue collected at the same time point. As shown in Fig. 3, elevations in IL-6 protein were detectable within the muscle homogenates at RTB in both diaphragm and soleus. These results demonstrate that IL-6 protein can be produced in vivo by skeletal muscle in the periods shortly after hyperthermia exposure.
Plasma IL-6, IL-10, IL-1β, and TNF-α protein expression during early and late HS recovery. Plasma samples were analyzed for changes in circulating IL-6, IL-10, IL-1β, and TNF-α cytokine expression to compare the systemic cytokine response to the local muscle response (Fig. 5). During TcMax, TNF-α (0.55-fold; P < 0.05) was significantly decreased. At RTB, IL-6 (4.27-fold; P < 0.01) was significantly increased and IL-1β (0.56-fold; P < 0.01) and TNF-α (0.44-fold; P < 0.01) were decreased. At HD, IL-6 (18.45-fold; P < 0.05) and IL-10 (3.16-fold; P < 0.05) were elevated and IL-1β (0.71-fold; P < 0.05) and TNF-α (0.49-fold; P < 0.01) were reduced. Overall, the circulating cytokine responses are marked by elevations in IL-6 and IL-10 and by reductions in TNF-α and IL-1β (Fig. 5). At 24 h there were no significant changes in circulating cytokines compared with matched controls. These systemic cytokine responses are similar to the mRNA and protein cytokine patterns observed in skeletal muscle during early recovery periods from HS (Figs. 2, 3, and 4).

TLR-2 and TLR-4 gene expression in HS and in early recovery. We evaluated the effects of HS on two of skeletal muscle’s dominant TLR isoforms, TLR-2 and TLR-4. As shown in Fig. 6A, TLR-2 mRNA was not affected by HS. However, in HS animals, TLR-4 mRNA expression was significantly increased in the diaphragm muscle (Fig. 6B, dark bars) at all time points, TcMax (2.29-fold, P < 0.01), RTB (1.81-fold, P < 0.05), and HD (3.2-fold, P < 0.05). Similarly, in the soleus (Fig. 6B, white bars), TLR-4 mRNA was significantly increased at TcMax (1.72-fold, P < 0.01), RTB (1.92-fold, P < 0.05), and HD (3.57-fold, P < 0.01). Thus during peak HS and throughout the early recovery phases in both the diaphragm and soleus, TLR-4 mRNA was significantly increased.

Expression of “early” transcriptional response genes to HS and in early recovery. Recently, we demonstrated that HSF-1 and the AP-1 regulatory elements within the IL-6 promoter are critical regulators of stress-induced IL-6 responses from in vitro myotubes (66). We used markers for both AP-1 and HSF activation, namely the transcriptional responses of c-fos, c-jun and HSP72, HSP25 to determine if upstream signaling systems to these pathways are activated during HS and early recovery. Results for c-fos and c-jun are shown in Fig. 7, A and B. Note the rapid transcriptional response and how their peak expression correlates with peak IL-6 mRNA (Fig. 2A). C-jun and c-fos mRNA were significantly increased throughout peak HS and the early recovery phase. In the diaphragm, c-jun and c-fos were upregulated at TcMax 3.82-fold (P < 0.01) and 3.23-fold (P < 0.05), at RTB 6.51-fold (P < 0.001) and 14.99-fold (P < 0.001), and HD 2.61-fold (P < 0.01) and 9.21-fold (P < 0.01), respectively. Similarly, in the soleus, c-jun and c-fos were upregulated at TcMax 5.41-fold (P < 0.01) and 25.11-fold (P < 0.01), at RTB 5.97-fold (P < 0.001) and 121.39-fold (P < 0.01), and at HD 2.07-fold (P < 0.05) and 29.55-fold (P < 0.01), respectively.

HSP72 (Fig. 8A) was upregulated, as expected, at TcMax (600.7-fold; P < 0.01) in the diaphragm and continued to be similarly elevated at RTB (951.95-fold; P < 0.01) and HD (776.14-fold; P < 0.01). The soleus muscle also exhibited great elevations in HSP72 mRNA at TcMax (229.98-fold; P < 0.001), which continued to rise at RTB (376.51-fold; P < 0.001) and HD (458.76-fold; P < 0.001) phases. Surprisingly,

Additional experiments and samples were used to determine if the pattern of cytokine transcripts were also synthesized into protein and stored within muscles at later time points. For these experiments, only diaphragm and gastrocnemius were large enough to measure multiple cytokines (IL-6, IL-10, IL-1β, and TNF-α), and so they are expressed separately in Fig. 4. At the HD recovery point, IL-6 protein did not quite reach significance (P = 0.05) in the gastrocnemius (Fig. 4B) and was absent in the diaphragm (as was shown in Fig. 3A). Interestingly, 24 h after the initiation of heat treatment, IL-6 (1.59-fold; P < 0.05), IL-10 (2.22-fold; P < 0.01), IL-1β (1.67-fold; P < 0.05), and TNF-α (1.74-fold; P < 0.05) were all elevated in the gastrocnemius but not in the diaphragm (Fig. 4D). The response of IL-10 was particularly robust at this time point. Overall, the responses seen at 24 h in the gastrocnemius were quite different from that of earlier recovery periods in terms of the pattern of cytokine expression and quite different from the diaphragm at the same time point.
HSP25 gene expression (Fig. 8B) never changed in the recovery period from HS in either diaphragm or soleus.

**Muscle histology.** Histopathology was performed on hindlimb muscles of six to seven mice to assess the extent of injury. The precise experimental parameters of these mice have previously been described (32). Representative photomicrographs of muscle at different phases of recovery are shown in Fig. 9. No significant abnormalities or lesions were detected at any time point.

**DISCUSSION**

The results demonstrate that skeletal muscle exhibits a pattern of gene expression in the early stages of recovery from HS that differs from the expected early responses to endotoxin by many systems, including skeletal muscle (4, 30, 71), cardiac muscle (30, 53), inflammatory cells (3, 49, 72), and in the circulation (23, 25, 26). The expected responses to endotoxin compared with the responses observed here are summarized in Fig. 10. First, the observed response is featured by early and predominant elevations in IL-6 and IL-10 mRNA and suppression or lack of significant stimulation of TNF-α and IL-1β, a pattern roughly parallel to cytokine measurements made in circulation in this study (Fig. 5) and as observed by others (5, 32, 52). The response is also characterized by stimulation of the TLR-4 gene in muscle, without stimulation of TLR-2, a pattern also reported to be closely aligned with direct effects of hyperthermia, when measured in inflammatory cells (74) and opposite of the acute effects of endotoxin on both inflammatory cells (41, 47) and skeletal muscles (30). Recent studies have demonstrated that TLR-4 is critical for long-term survival in heat stress (10).

During the late recovery period (24 h) the gastrocnemius muscle produced a different kind of inflammatory response, which included the upregulation of protein levels of TNF-α, IL-1β, and IL-6 and notably marked elevations in IL-10. Interestingly, this pattern was not seen in the diaphragm in this time window, nor was it seen for any other muscle at any time point, an observation for which we have no clear explanation. Differences in muscle phenotype or contractile activity are plausible explanations for which there is some support (51, 63). This 24-h time point is interesting because the pathology of our HS model results in a fever-like state at ~24 h post TcMax (33), as evidenced by an elevation in core temperature (Fig. 1 and Table 1) (28, 35, 37). At least two possible explanations exist for this late response. First, at this time point, muscle may be responding to late-phase elevations in PAMPs or DAMPs that stimulate TLRs to induce this type of cytokine profile (30, 42). Alternatively, heat treatment could result in an acute muscle injury (i.e., rhabdomyolysis), followed by delayed infiltration of inflammatory cells that produce cytokines, normally functioning in repair and regeneration (62). Specifically, the presence of TNF-α and IL-10 during recovery from injury is critical for normal muscle repair (11, 64). However, muscle cross sections stained with hematoxylin and eosin from C57BL/6J mice that underwent a more severe HS protocol with an end point of Tc = 42.7°C revealed no heat-induced damage at any point up to 24 h after HS (Fig. 9). Therefore the most likely explanation for the late phase signal is activation of TLRs within the muscle fibers.

The findings support the postulate that the pathology of HS results in two distinct cytokine signaling events in muscle. The first is an early stress response, stimulated by hyperthermia, or secondary signals such as protein stress or oxidative stress. These events may be shared by other parenchymal tissues as well. This early response could serve a preconditioning function, preparing muscle to increase its capability to withstand...
The distinct cytokine profile (Fig. 2) and the absence of damage (Fig. 9) in muscle during the early recovery phase from HS does not support the presence of endotoxin or the early invasion of inflammatory cells as the underlying stimulus of HS-induced muscle cytokine production (2, 15, 62, 71). In contrast, the late phase cytokine response of the gastrocnemius is likely to reflect a response to PAMPs or DAMPs via TLR activation, where elevations in TNF-α (Fig. 4D) as well as the other cytokines involved with innate immunity are concurrent (30).

Evidence of early activation of transcription factors regulating IL-10 and IL-6. Both IL-6 and IL-10 are regulated by complex networks of transcription factors; see recent reviews (48, 65). Recently, we have identified HSFs as an element of IL-6 transcriptional regulation in muscle (66, 67). Inhibition of HSF-1 attenuated hyperthermia-induced IL-6 mRNA upregulation. Interestingly, IL-10 is also under HSF-1 regulation in inflammatory cells (73), but the pathways are not yet understood in muscle and its molecular regulation is highly phenotype-specific (55). In these in vivo muscles, activated HSFs are no doubt greatly elevated at very early time points, based on the observation that HSP72 mRNA was already increased by several orders of magnitude at TcMax (Fig. 6).

Interestingly, we expected the small molecular weight HSP25 to be upregulated as well. Most other studies, from a variety of tissues, have shown that these two HSPs are simultaneously upregulated in hyperthermia (27). In fact, we have previously reported HSP25 mRNA to be upregulated in mice that underwent a more severe HS treatment (34). Therefore we suggest that the HSP25 gene may require a greater hyperthermic stimulation to be upregulated. Other investigators have also reported differential responses of HSP72 and HSP25 following hyperthermia in heart muscle (31) and differential regulation of other HSPs following hyperthermia has also been reported (22). HSP25 is known to exert a feedback inhibition of HSF-1 transcriptional activity (7). One plausible explanation is that this feedback loop could be differentially affected during different stress conditions.

Another common regulatory component of the “early” intracellular signaling of innate immunity is the activation of MAPKs (68). MAPKs (p38, JNK and ERK1/2 families) are activated by phosphorylation or dephosphorylation, in response to stress-associated signals that are present in hyperthermia. MAPKs interact with large networks of signaling molecules, but among the earliest indicators of their activation is an increase in both c-fos and c-jun transcription, a response categorized as the “early response genes” to stress (57). This is of particular importance with regard to IL-6 and IL-10 regul-
lation (48, 54) because c-fos and c-jun dimerize and bind to AP-1 regulatory elements on the DNA of both cytokines. We have previously shown that the AP-1 response element is a critical component of IL-6 transcriptional regulation during hyperthermia in myotubes (66).

The early and pronounced upregulation of TLR-4 that was seen during HS and early recovery implicates TLR-4 as an other plausible mechanism for the induction of the cytokine stress response in mice during and after HS. Endogenous ligands such as HMGB1 are rapidly upregulated in plasma at the onset of HS and throughout recovery. HMGB1 signals through TLR-4 and is an important mediator of inflammation, injury, and mortality in HS (10, 56). We have previously measured HMGB1 gene expression in C2C12 myotubes following heat shock (42°C 1 h) and saw no changes (unpublished observations). These findings do not preclude HMGB1 from being an important mediator of the cytokine response seen here but suggest that HMGB1 may be a paracrine or endocrine signal from another cell type that initiates the stress response of skeletal muscle or other parenchymal tissue. Nevertheless, the pattern of cytokine responses we observed in the early stages of recovery are not consistent with TLR-4 activation by HMGB1 (2, 15), so it is more likely that responses to HMGB1, if present, emerge at later time points.

Certainly other gene regulatory networks could be responsible for an early innate immune response in skeletal muscle; most notably, both IL-6 and IL-10 are transcriptionally activated by NF-kB activation, reviewed in (55, 65). However, it is unlikely that heat exposure alone works through this pathway, because inducible HSPs, a product of heat shock, inhibit NF-kB pathway activation (9). Additionally, we have shown

![Fig. 7. Responses of the early MAPK response genes, c-fos and c-jun to HS.](image)

c-jun mRNA \( (A) \) was rapidly elevated at \( T_{cMax} \) and remained elevated throughout the early recovery period in diaphragm (dark) and soleus (white); \( T_{cMax} \), RTB, HD. Similar findings were observed for c-fos \( (B) \), with a more predominant fold change than seen for c-jun. Values are mean fold change calculated using the \( \Delta \Delta CT \) (threshold cycle) method, with the range of fold change below each bar. Control gene expression was similar among all groups, assigned a value of 1, and illustrated as a dashed line \( (T_0) \) for presentation purposes. Sample size was 6–7 mice/group. Comparison HS vs. controls at each time point; \* \( P < 0.05 \), \** \( P < 0.01 \), \*** \( P < 0.001 \). For soleus vs. diaphragm at each time point \# \( P < 0.01 \), \## \( P < 0.01 \).}

![Fig. 8. Responses of heat shock protein mRNA message to HS.](image)

HSP72 mRNA \( (A) \) was rapidly elevated at \( T_{cMax} \) and remained elevated throughout the early recovery period in diaphragm (dark) and soleus (white); \( T_{cMax} \), RTB, HD. No changes in HSP25 \( (B) \) mRNA response during HS-recovery. Values are mean fold change calculated using the \( \Delta \Delta CT \) (threshold cycle) method, with the range of fold change below each bar. Control gene expression was similar among all groups, assigned a value of 1, and illustrated as a dashed line \( (T_0) \) for presentation purposes. Sample size was 6–7 mice/group. Comparison HS vs. controls at each time point; \* \( P < 0.05 \), \** \( P < 0.01 \), \*** \( P < 0.001 \). For soleus vs. diaphragm at each time point \# \( P < 0.01 \), \## \( P < 0.01 \), ### \( P < 0.001 \).
that pharmacological inhibition of NF-κB had no impact on IL-6 gene expression in response to hyperthermia in myotubes within the early time frame of 1–3 h (67). We conclude that at least two elements of both IL-6 and IL-10 regulation (HSF and c-fos, c-jun) are activated very early in the response to HS and are capable of providing a substantial component of the transcriptional message necessary to induce an early stress-induced immune response.

Limitations to the experiment. There are several important limitations to this study. First, we cannot conclusively state that skeletal muscles are responsible for a significant component of the typical early circulating cytokine profile commonly seen in HS. That experiment is an extremely challenging one to perform because of the ubiquitous presence of skeletal muscles throughout the body. We can say that the results parallel the responses seen in the circulation and that due to the great mass of muscle (~40% of body mass in man, ~28% in mouse) skeletal muscle may be an early contributor to whole organism stress responsiveness. It is likely that other parenchymal cell types are also involved in this period.

Another limitation of this study is that intracellular protein cannot be used to reflect secretion rates. For example, elevations in resident protein can mean that secretion is inhibited, if protein synthesis is preserved. Unlike IL-1β and TNF-α (which can be stored in pre- or transmembrane forms and then enzymatically cleaved and released), IL-6 and IL-10 are not stored in the precursor form.

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*Fig. 9.* Photomicrographs of muscle exposed to heat stroke. Representative photomicrographs of hindlimb muscle from C57BL/6J male mice at (A) control, (B) T\text{max} of 42.7°C, (C) at hypothermia depth, and (D) at (A) T\text{max}, (B) 24 h. Tissues were stained with hematoxylin and eosin for microscopic evaluation at magnification X200. Adjustments to color and contrast were applied to all four images simultaneously using MS PPT software.

*Fig. 10.* Proposed pathways for muscle immune responses during heat stroke. A theoretical model distinguishing expected responses to TLR activation (left, hypothesis H1) vs. direct protein stress signaling (right, Hypothesis 2). Left: toll-like receptor (TLR) activation in muscle and other tissues has previously demonstrated an early TNF-α and IL-1β response. These could promote a delayed autocrine IL-6 and IL-10 response based on known transcriptional regulation pathways. Right: the proposed stress-induced immune response predicts that both IL-6 and IL-10 are directly stimulated by stress signaling. The observed inhibition of TNF-α and IL-1β, downstream of IL-6 and IL-10, are known signaling pathways. The HSF and stress activated protein kinase (SAPK) pathways are components of stress-induced IL-6 signaling and were indirectly tested in this study by measuring mRNA biomarkers, HSP72, c-fos, and c-jun.
stored in a preform but are synthesized for secretion by one of several pathways, most likely involving vesicular transport (29, 40, 58). Little is known about the secretory pathways for cytokines in skeletal muscles. In our experience, in response to heat exposure, large elevations in IL-6 secretion can be observed from skeletal muscle myotubes with no concomitant rise in intracellular protein in the lysate, suggesting that there is very little storage of synthesized IL-6 protein. Nevertheless, the elevation in IL-6 at RTB (~32 min post T_{Max}) confirms that IL-6 synthesis is ongoing during this very early time frame.

Furthermore, in some experiments the gastrocnemius was used rather than the soleus because of experimental limitations. The use of different muscles means the introduction of different muscle phenotypes. Although, we expect muscles of the hind limb to experience similar environmental conditions, these phenotypes may respond differently. Thus assigning significance to results from different experimental muscle groups in this study should be done with caution.

CONCLUSIONS

The data are consistent with a working hypothesis that skeletal muscles (and likely other parenchymal cells) participate in the early response to an acute stress event, possibly responding directly to hyperthermia (65). We have titled this response a “stress-induced cytokine response,” but other components of the immune system such as TLRs are likely to be responding as well, possibly in a different time frame. Whether this outcome represents a local autocrine/paracrine defense mechanism or a true endocrine role for skeletal muscles for the integrated response of the organism remains to be determined. The pattern of cytokine expression may function to suppress subsequent proinflammatory signaling locally or systemically, as the anti-inflammatory effects of IL-6 include suppression of both TNF-α (59) and IL-1β (70) and stimulation of anti-inflammatory IL-1ra and IL-10 (60). IL-10 is also well known for its ubiquitous anti-inflammatory influences on both immune cells and tissues. Together, these responses could play critical roles in dampening proinflammatory pathways, in initiating survival programs, preconditioning, or initiating secretion of acute phase proteins from the liver (1, 38, 43, 44). At a local level, production of IL-6 and IL-10 may protect muscle fibers from subsequent stress exposure (45, 62). By protecting the integrity of muscle fibers, multiple organ systems may likewise be protected from heat-induced rhabdomyolysis, a common feature of HS that contributes to injury in multiple organs, particularly the kidney (24). In later stages of recovery from HS (i.e., at 24 h) this early response in limb muscle may shift to a more classic innate immune response, possibly induced by a late presence of endotoxin, effects of other inflammatory mediators, or because upregulation of TLR-4 may make muscle more sensitive to low levels of inflammatory mediators.

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DISCLOSURES

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.

AUTHOR CONTRIBUTIONS

Author contributions: S.S.W. and L.R.L. conception and design of research; S.S.W., S.M.D., and L.R.L. performed experiments; S.S.W., T.L.C., S.M.D., and L.R.L. analyzed data; S.S.W., T.L.C., and L.R.L. interpreted results of experiments; S.S.W. prepared figures; S.S.W. and T.L.C. drafted manuscript; S.S.W., T.L.C., S.M.D., and L.R.L. edited and revised manuscript; S.S.W., T.L.C., S.M.D., and L.R.L. approved final version of manuscript.

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