Hyperthermia increases interleukin-6 in mouse skeletal muscle

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Am J Physiol Cell Physiol 303: C455–C466, 2012. First published June 6, 2012; doi:10.1152/ajpcell.00028.2012.—Skeletal muscle produces and contributes to circulating levels of IL-6 during exercise. However, when core temperature is reduced, the response is attenuated. Therefore, we hypothesized that hyperthermia may be an important and independent stimulus for muscle IL-6. In cultured C2C12 myotubes, hyperthermia (42°C) increased IL-6 gene expression 14-fold after 1 h and 35-fold after 5 h at 37°C recovery; whereas exposure to 41°C resulted in a 2.6-fold elevation at 1 h. IL-6 protein was secreted and significantly elevated in the cell supernatant. Similar but reduced responses to heat were seen in C2C12 myoblasts. Isolated soleus muscles from mice, exposed ex vivo to 41°C for 1 h, yielded similar IL-6 gene responses (>3-fold) but without a significant effect on protein release. When whole animals were exposed to passive hyperthermia, such that core temperature increased to 42.4°C, IL-6 mRNA was elevated 5.4-fold compared with time matched controls. Interestingly, TNF-α gene expression was routinely suppressed at all levels of hyperthermia (40.5–42°C) in the isolated models, but TNF-α was elevated (4.2-fold) in the soleus taken from intact mice exposed, in vivo, to hyperthermia. Muscle HSP72 mRNA increased as a function of the level of hyperthermia, and IL-6 mRNA responses increased proportionally with HSP72. In cultured C2C12 myotubes, when heat shock factor was pharmacologically blocked with KNK437, both HSP72 and IL-6 mRNA elevations, induced by heat, were suppressed. These findings implicate skeletal muscle as a “heat stress sensor” at physiologically relevant hyperthermia, responding with a programmed cytokine expression pattern characterized by elevated IL-6.

tumor necrosis factor-α; heat stroke; myokines; cytokines; exercise; acute stress; heat shock protein 72; heat shock factor

MUSCLE CYTOKINE PRODUCTION has largely been viewed in the context of exercise. Early observations linked the elevations of circulating cytokines (12) with inflammatory cell activation due to muscle injury or less well-defined inflammatory mediators such as endotoxin in the blood (15). However, in recent years, a paradigm shift has occurred with the observation that during exercise muscle can behave as an endocrine organ, producing significant amounts of cytokines such as interleukin-6 (IL-6) and others (53, 67). Muscle is not just a downstream effector of exercise-related circulating factors, it can also be locally stimulated to produce cytokines with contraction (24, 49, 54, 67). In addition, skeletal muscle tissue and cells have been shown to be responsive to stimuli that are not necessarily exercise related, including: endotoxin (17), inflammatory cytokines (16, 43, 70), catecholamines (17, 25, 36), low glycogen (33, 66), ATP (11), intracellular Ca2+ (2, 30), surgical manipulation (60), reactive oxygen species (ROS) (35), and nitric oxide (NO) (44, 68). Most of these stimuli occur during conditions of “stress” at either the tissue or the whole organism level.

Previous studies have demonstrated some interactions between core body temperature and IL-6 production during exercise. For example, when core temperature is decreased during endurance exercise in humans the IL-6 response is attenuated (56), and when endurance exercise is performed in a heated environment the circulating IL-6 response is augmented (65), suggesting that the skeletal muscle IL-6 response is a temperature-related phenomenon. In addition, IL-6 is one of the most consistent and highly expressed circulating cytokines following heat exposure and heat stroke (7, 8, 40, 57). For example, in humans, therapeutic hyperthermia, which is used for cancer treatment (41.8°C core temperature for 30 min), results in striking elevations of IL-6 in the absence of heat illness (57). The role of IL-6 in this context is not well understood. However, IL-6 knockout animals have a greater morbidity to acute hyperthermia exposure (41), and IL-6 has the potential to suppress local and systemic inflammatory responses by reducing expression of proinflammatory cytokines, interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) (59, 73). It is also the principal mediator of the acute phase inflammatory response, an important component of the hyperthermia response resulting in production of protective acute phase proteins (20). In this context it is often considered a proinflammatory mediator. Its actions are truly complex.

The cellular source of the IL-6 and other circulating cytokines seen during and after hyperthermia is also unknown. However, since skeletal muscle is capable of producing significant levels of cytokines in exercise and previous studies have shown that core temperature during exercise influences circulating IL-6 (56, 65), we hypothesized that hyperthermia may be an additional and unrecognized stimulus for muscle IL-6 production. Through the use of in vivo, ex vivo, and cell culture models, we provide evidence to support this hypothesis, establishing that physiologically relevant acute heat exposure, within the typical range of muscle temperatures during exertional hyperthermia, high fever, or severe heat illness (41–42°C), is a novel stimulus for muscle IL-6. In addition, we explore the influence of hyperthermia on muscle TNF-α and other cytokines and chemokines produced by skeletal muscle cells.

MATERIALS AND METHODS

Anesthetized heat stroke model. Male C57BL6 mice, 3–4 mo old, were purchased from Jackson Laboratories (Bar Harbor, ME) and...
housed at the University of Florida. All animal housing and procedures were performed at The University of Florida and were approved by The University of Florida’s Institutional Animal Care and Use Committee. A total of 18 adult mice were given ad libitum access to food and water prior to experimentation. Animals were anesthetized with =0.3 ml ip of a 10% pentobarbital solution (Neumubtal); 0.05 ml ip supplements were given, as needed. A 0.5 ml supplement of sterile saline was given before we commenced temperature-specific treatment to prevent dehydration and circulatory collapse in the mice during the extended experiments. No further hydration supplements were provided. Core temperature was monitored using a YSI 401 rectal thermistor connected to a temperature monitor/PID servo-control unit (Digi-sense). Data were recorded in 15 s intervals with Digi-sense software (Eutech). Core temperature was normothermic, maintained at 37°C, or incrementally elevated over ~3.5 h to a peak of 42.4°C. This peak corresponds to the core temperature required to induce mild heat stroke in anesthetized mice, as described by Leon et al. (40). The method of temperature elevation was achieved with a protocol described in detail previously (50). In brief, temperature was initially elevated to 39.5°C for 30 min by a long wavelength infrared heat lamp (4–14 μm) and then was incrementally increased 0.5°C every 30 min until the target peak was achieved for one 20 s sample period. This was followed by a 30 min recovery period, during which core temperature was allowed to return to 37°C. Some additional experiments were performed with a recovery period of 2 h to follow plasma cytokine production. At the conclusion of either protocol a cardiac puncture was performed to collect blood, and the soleus was harvested and stored at -80°C for later analysis.

Ex vivo muscle preparation experiments. Soleus muscles from three groups of mice were studied in oxygenated muscle baths to understand the influence of heat exposure on muscle mRNA and protein release. In group 1 (n = 8), mice were asphyxiated with CO₂, and the soleus muscles quickly removed and placed in oxygenated Krebs Ringer solution at 22°C, containing (in mM) 121 NaCl, 5 KCl, 1.8 CaCl₂, 0.5 MgCl₂, 0.4 NaH₂PO₄, 24 NaHCO₃, 5.5 glucose, 0.1 EGTA, 10 μM D-Tubocurarine. The buffer was further dosed with 10 μg/ml polymyxin B sulfate (Calbiochem) to eliminate the effects of potential contamination with endotoxin in the open baths. This dose was shown to block LPS-induced activation of Toll-like receptor (TLR)-4 (23). Muscles were continuously bubbled with 95% O₂/5% CO₂ asphyxiation (n = 8). The soleus muscles were then prepared at room temperature in oxygenated buffer identical to that described for group 1. However, a 4 ml standard muscle bath (Radnoti) was used, and L₀ was adjusted to a standard preload of 1 g, without stimulation. The muscles then underwent an identical protocol as in group 1, but without any electrical stimulation at any time. An additional subgroup of animals (n = 6) was studied at this time, in which soleus muscles were rapidly removed from newly anesthetized animals and flash-frozen for later mRNA analysis. These tissues were used to estimate the changes in IL-6 and TNF-α mRNA message, relative to housekeeping genes, in both groups 1 and 2.

To determine the influence of heat exposure on contractile function, a third group of isolated soleus muscles was studied (group 3, n = 4). These were put through a nearly identical protocol as group 2, but force frequency curves were generated at baseline and after the total 2 h experimental period. As before, one soleus was exposed to continuous 37°C for 2 h and the matched soleus from the same animal was exposed to 41°C for 1 h, followed by recovery at 37°C for 1 h. Cell culture. The C2C12 mouse myoblast cell line was purchased from the American Type Culture Collection (Manassas, VA). Laboratory cell stocks were routinely tested for mycoplasma infection using the method described by Zakharova et al. (76). Cells were grown in six-well cell culture plates (Corning, Corning, NY), 2 ml medium volume, and cultured in Dulbecco’s modified Eagle’s medium (Mediatech, Manassas, VA) containing 10% standard fetal bovine serum (HyClone, Logan, UT). Cells were grown to 70–80% confluence in a water-jacketed humidified incubator with 5% CO₂ set at 37°C (NAPCO 8000WJ, Thermoscientific, Marietta, OH). Experiments were performed using both myoblasts and myotubes. For preparation of myotubes, the medium was exchanged with fresh medium containing 2% donor equine serum (HyClone) and allowed to differentiate for 5 days into multinucleated fibers. All cultures received fresh media 18 h preceding the heat treatment. A second water-jacketed humidified incubator with 5% CO₂ was preset to an environmental temperature (T_ENV) of 42, 41, or 40.5°C (Forma Scientific 3154). T_ENV within the incubator was monitored using an YSI thermistor, accurate to 0.01°C. Once myoblasts and myotubes were placed in the incubator, it took ~33 min for the T_ENV to reach 42°C; this steady temperature was then retained for 60 min. Cells were harvested either immediately after heat treatment (1 h) or after recovery periods at 37°C of 1 h, 2 h, and 5 h. Control cells (CON) were treated identically but kept at 37°C for the entire experiment. Measurements of medium temperature in the baths that were heated required ~10–15 min delay of return of the medium temperature to 37°C. In some experiments the cultured cells were treated with heat shock factor (HSF) inhibitors, ATP receptor inhibitors, or NF-kB inhibitors prior to exposure to heat. Both sham controls with solvent vehicles and unheated cells treated with these agents were tested simultaneously. The doses and procedures for these agents are described in RESULTS and in the figure legends.

For mRNA measurements, cells were lysed in RNA-Isol Lysis reagent (5 Primer) and immediately stored at -80°C. Additionally, the supernatant for the CON and 6 h samples were stored at -80°C for later multiplex protein analysis or lactate dehydrogenase (LDH) determination. Additional experiments using myotubes were performed at T_ENV of 40.5 and 41°C.

RNA isolation and real-time PCR analysis. Cells were harvested and lysed in RNA-Isol Lysis Reagent according to the manufacturer’s instructions.Briefly, RNA was separated from protein and DNA by the addition of bromochloropropane and precipitation in isopropanol. After a 75% ethanol wash and resuspension in DEPC H₂O, purity of RNA samples was quantified by spectrophotometry. Total mRNA (1 μg) was then reverse transcribed using Verso cDNA Synthesis Kit (Thermo Scientific). Preformulated TaqMan Gene Expression Assays were purchased from Applied Biosystems for the following mouse genes: IL-6 (Mm00446191_m1), glyceraldehyde-3-phosphate (GAPDH) (Mm99999915_s1), heat shock protein 1A (HSP72) (Mm01159846_s1), β-actin (ACTB) (Mm00607939_s1), hypoxanthine guanine phosphoribosyl transferase (HPRT) (Mm01545399_m1), C456 IL-6 AND OTHER MYOKINES PRODUCED IN HEAT STRESS
and TNF-α (Mm00443258_m1). Relative quantitative real time reverse-transcription polymerase chain reaction (RT-PCR) was performed using the TaqMan Gene Expression Master Mix (Applied Biosystems), and reactions were performed in duplicate using 96-well optical plates on a StepOnePlus Real-Time PCR System (Applied Biosystems). Each target passed a validation experiment, thus the efficiencies between target and endogenous controls were approximately equal (<0.1). Candidates for housekeeping genes, GAPDH, HPRT, and ACTB, were tested for stability over various experimental treatments (data not shown). GAPDH was used as the endogenous control to normalize the samples; GAPDH has been a commonly used stable housekeeping gene with C2C12 cells (18, 19).

Relative quantitation was done using the ΔΔCT method, where CT is the threshold cycle, and all untreated samples were normalized to 1.

Cytokine assay. Measurements of cytokines and chemokines [G-CSF, GM-CSF, IFN-γ, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IP-10, KC, MCP-1, MIP-1α, RANTES, TNF-α], and soluble receptors (sGP130, sIL-6R, sTNFR1, sTNFR2, sIL-1R1, and sIL-1R2) were performed on cellular supernatant samples and from EDTA-treated plasma samples from the anesthetized hyperthermic mice. The platform used was the MILLIPLEX MAP Mouse Cytokine/Chemokine - Premixed 22-Plex Assay and a custom Mouse Soluble Cytokine Receptor Panel (Millipore, Billerica, MA). The tests were performed according to the manufacturer’s protocols. In brief, supernatant and antibody-coated beads were added to a 96-well primed filter plate and allowed to incubate overnight at 4°C. Following three washes, biotinylated detection antibodies were allowed to incubate for 1 h at room temperature, after which SAV-phycoerythrin was allowed to incubate for 30 min at room temperature. All incubations occurred during gentle shaking. Following three washes, beads were resuspended in sheath fluid and reactivity acquired using a Luminex 200 IS system with Xponent software (Millipore). Concentrations in pg/ml were determined using a standard curve, five-parameter logistics and Milliplex analyst software (Viagene). Acquisition and analysis has been optimized for multiple parameter measurements.

IL-6 ELISA and LDH assays. A mouse IL-6 ELISA (eBioscience) was used to quantify IL-6 released into the supernatant of ex vivo muscle bath and cell culture experiments. Briefly, frozen samples of physiological buffer from in vitro experiments were brought to room temperature and the samples were placed on a microwave plate coated with a monoclonal anti-mouse IL-6 antibody. A biotin-conjugated anti-mouse IL-6 antibody was added to bind to mouse IL-6 captured by the plate-coated antibody. Streptavidin-horseradish peroxidase (HRP) was added to bind to the biotin-conjugated anti-mouse IL-6 antibody. A substrate solution, reactive with HRP, was then added to the wells, and a colored product was formed in proportion to the amount of mouse IL-6 present. LDH activity assays were performed to evaluate cytotoxicity in the cell culture experiments. We tested 100 μl samples of cell supernatant using a commercially available kit (Cayman Chemical) per the manufacturer’s instructions.

Statistics. Values of central tendency were expressed as means ± SE for normally distributed data and, when noted, were expressed as medians for skewed distributions. We usually expressed data as a log function, base 2, to be able to highlight the treatment effects, which often covered a broad range of response, and/or to transform skewed data to more normal distributions. Unless otherwise noted, the 42°C cellular experiments were repeated on 3 separate days with six samples taken from each day. The 40.5°C and 41°C cell experiments, as well as the signaling inhibitor experiments, were performed on 4 separate days with two samples taken each day. For anesthetized heat stroke studies there were n = 7 for matched controls and n = 11 for heat stroke animals. For ex vivo animal studies, two groups of eight total mice were used for paired CON and HEAT samples and four mice were used for contractile experiments (total n = 12). Samples for RT-PCR were run in duplicate and analyzed by multiway ANOVA; post hoc analysis was performed by comparing individual means using mean contrasts (SAS JMP software). Nonparametric statistics (Wilcoxon or Kruskal-Wallis for multiple groups) were used on sample populations that were not normally distributed. A minimum statistical significance was set at a P < 0.05.

In cytokine/chemokine, multiplex analysis (Luminex), all targets were evaluated for outliers, using Grubb’s outlier test (69). If one outlier was found per experimental group then that outlier was removed from analysis; if multiple outliers were found, then all data were included. Furthermore, target proteins that were predominantly found to be within normal detectable limits were excluded. Each target, with consideration to its experimental group, was tested for normal distribution. If not normally distributed, nonparametric Wilcoxon tests were used to determine P values for differences between groups. P values for all 15 targets were adjusted by the Benjamini-Hochberg procedure to control the false discovery rate (FDR) (5). FDRs of 0.15, 0.10, and 0.05 were compared and reported where appropriate.

RESULTS

Response of cultured skeletal muscle cells to acute hyperthermia. Mean IL-6 mRNA expression was elevated ∼4-fold in myoblasts and ∼14-fold in myotubes, immediately following 1 h of 42°C exposure (Fig. 1A). Surprisingly, in myotubes, but not myoblasts, IL-6 mRNA expression was at its greatest level at 35-0.10, and 0.05 were compared and reported where appropriate.

and TNF-α mRNA was no longer upregulated but, rather, decreased at 2 h postheat. To test whether these effects could have been a reflection of cell damage from the heat exposure, LDH activity was measured in the media from the muscle baths in heated and sham control experiments. The distribution of LDH measurements from the heated experiments was nonparametric. The medians were 1.33 in control vs. 1.83 μU/mg lysate protein in heated baths (n = 8 independent samples/ group, P = 0.56), suggesting no significant cell damage due to 1 h of 42°C heat treatment and recovery (data not shown).

Peak HSP72 mRNA was observed at 1 h postheat (Fig. 1C). As with IL-6 (Fig. 1A) and TNF-α (Fig. 1B), the HSP72 response was greatest in myotubes. HSP72 mRNA was up regulated ∼1,400- and 400-fold in myotubes and myoblasts, respectively. The HSP72 responses were transient; within 5 h after heat, HSP72 mRNA was no longer significantly different from baseline.

As shown in Fig. 2, a lower level of hyperthermia (41°C) also elicited an IL-6 response and significantly inhibited TNF-α mRNA, being qualitatively similar but at a lower magnitude than the responses to 42°C (Fig. 1). IL-6 mRNA was increased ∼2.25- and ∼2.65-fold immediately following heat exposure (1 h) and 1 h into recovery (2 h), respectively. TNF-α mRNA was decreased ∼2.5-fold immediately following heat (1 h). HSP72 mRNA was also increased ∼14.5- (1 h), ∼10.5- (2 h), and ∼5.8-fold (3 h). Following 40.5°C (Fig. 2A) exposure, IL-6 mRNA was no longer upregulated but, rather, downregulated slightly ∼1.45- and ∼2.15-fold following 1 and 2 h (3 h) of recovery. However, TNF-α and HSP72 mRNA behaved qualitatively the same as they did after 41°C; i.e., TNF-α mRNA decreased ∼2.2-fold (1 h) and HSP72 mRNA increased ∼9.65- (1 h), ∼5.95- (2 h), ∼2.5-fold (3 h).

Effects of hyperthermia on protein production in supernatants of C2C12 cells. Multiplex kits were used to measure protein expression released into the C2C12 culture superna-
tants in response to heat. These are the same experimental series in which mRNA was measured (Fig. 1), but samples were only obtained after a 5 h recovery period, thus representing accumulated protein over 18 h. Therefore, the values measured after 1 h of heat and 5 h of recovery are over and above the accumulated protein arising from 18 h of incubation at 37°C. Figure 3 shows absolute concentrations within the supernatants for both IL-6 and TNF-α. IL-6 was significantly elevated in myotube culture media, but IL-6 protein expression was orders of magnitude lower in myoblasts and often below the detection limit of the assay (Fig. 3A). In contrast, TNF-α protein production was undetectable in nearly all experiments.

Figure 4 compares changes in the remaining cytokines, chemokines, and soluble cytokine receptors measured with the Luminex assay. Changes are expressed as the “average difference” between samples from heated cells and the mean protein concentration of the unheated control cells. Only samples in which over half of the measurements exceeded the “minimum detectable concentration” and showing at least a 0.1-fold change are shown. Samples that were downregulated are shown on the left (white vertical bars) and upregulated on the right (dark shaded vertical bars). In myoblasts (Fig. 4A), only IP-10 of the 22 cytokines and chemokines was significantly upregulated in response to heat. In myotubes (Fig. 4B), similar elevations of the same chemokines were seen in myoblasts but none were statistically significant. Using a separate soluble

**Fig. 1.** C2C12 myoblast and myotube IL-6 (**A**), TNF-α (**B**), and HSP72 (**C**) mRNA responses over time, following 42°C for 1 h. Myoblasts (○) and differentiated myotubes (□) were grown as described in METHODS, maintained at 37°C or exposed to 42°C for 1 h, and harvested immediately (1 h) or allowed to recover for 1 h (total 2 h), 2 h (3 h), or 5 h (6 h). Statistical tests were post-ANOVA least squares contrasts between means. Results are from multiple cultures in 3 independent experiments.

**Fig. 2.** Response of C2C12 myotubes to lower levels of hyperthermia, 41°C and 40.5°C 1 h. Differentiated C2C12 myotubes [IL-6 (**A**), TNF-α (**B**), and HSP72 (**C**)] were treated as shown in Fig. 1, except heat exposure was at either 41°C (solid lines) or 40.5°C (broken lines). Statistical tests were post-ANOVA least squares contrasts between means. Results are from 2 samples each in 4 separate independent experiments. *P < 0.05, **P < 0.01, ***P < 0.0001.
cytokine receptor kit (Fig. 4C) we also tested myotube supernatants for a variety of soluble receptors. Only soluble (s) TNFR2 reached statistical significance and was downregulated by heat exposure. In summary, of the many cytokines studied, IL-6 was the only cytokine significantly affected by heat. Of the chemokines and soluble receptors, only IP-10 in myoblasts and sTNFR2 in myotubes were significantly impacted by heat exposure.

Ex vivo experiments: comparison of IL-6, TNF-α, and HSP72 mRNA isolated mouse soleus. To test whether similar heat exposures induce IL-6 and TNF-α responses in intact muscles, soleus muscles were isolated and studied in tissue baths ex vivo. In group 1, both soleus muscles were rapidly excised from mice asphyxiated with CO2. In this experimental group the muscles were stimulated with twitch contractions during adjustment of L0, and were also stimulated with 1 twitch/min throughout the remaining protocol. The effects of treatment are seen in Table 1. Two comparisons are made. First, mRNA expression, as measured at the end of the experiments, was compared with expression in fresh untreated soleus (using the background housekeeping genes as a reference). An unexpected finding was that isolation and incubation of soleus muscles at 37°C bath temperature for 2 h resulted in a >70-fold elevation in mRNA for IL-6, and a >7-fold elevation in TNF-α mRNA; whereas HSP72 mRNA was unaffected. One hour exposure at 41°C hyperthermia and 1 h of recovery at 37°C resulted in a significant and nearly threefold additional elevation in IL-6 mRNA, which was ~210-fold above fresh soleus. The baseline TNF-α mRNA was nearly halved by heat treatment. HSP72 was stimulated 45-fold by heat.

Because of the unexpected results in Group 1 studies, new experiments (group 2) were done to try to eliminate the background elevation in IL-6 mRNA. These animals were anesthetized during isolation, thus allowing the muscles to be perfused right up until the final extraction step. Furthermore, these muscles were never stimulated before or during the protocol, they were stretched to an “estimated” L0 (1 g) and were studied in a small but standard tissue bath. Again, isolation and exposure to the ex vivo conditions of the muscle bath resulted in a >35-fold elevation in IL-6 mRNA and similar responses of both TNF-α and HSP72 as were seen in group 1. The effects of heat exposure were qualitatively similar to group 1 compared with matched controls, i.e., 1 h of heat treatment elevated IL-6 mRNA nearly fourfold, over and above the strong background effect of muscle isolation.

IL-6 protein release from the muscle was studied from the buffer solutions at the end of the ex vivo experiments, Fig. 5A. In group 1, the sham control tissue baths contained an average of 152 ± 27 pg/ml at the end of the experiment, a value that represents ~30 pg/mg of muscle/h, assuming an average soleus weight of 10 mg. Although 1 h exposure at 41°C resulted in a small elevation in IL-6 protein in five of seven tissue pairs, this did not reach statistical significance (P = 0.14). In con-
controls measured at 2 h of 37°C.

stimulated at 1 twitch/s and exposed to 1 h 41°C followed by 1 h 37°C. Sham protein release from isolated soleus muscles.

Fig. 5. Ex vivo soleus protein and contractile function.

A  Protein secretion

B  Force Frequency - Controls

C  Force Frequency - Heat

Table 1. Effects of muscle isolation and heat treatment on IL-6, TNF-α, and HSP72 mRNA

<table>
<thead>
<tr>
<th></th>
<th>Fresh Soleus</th>
<th>Sham Control (2 h 37°C)</th>
<th>Heat (1 h 42°C + 1 h 37°C)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Group 1</td>
<td>Group 2</td>
<td>Group 1</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.0 ± 0.4</td>
<td>72.7 ± 15.2**</td>
<td>210.1 ± 28.0**</td>
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<tr>
<td></td>
<td></td>
<td>35.3 ± 9.4**</td>
<td>126.6 ± 12.1**</td>
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<tr>
<td>TNF-α</td>
<td>1.0 ± 0.2</td>
<td>7.2 ± 1.4**</td>
<td>3.5 ± 0.7**+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.5 ± 2.2**</td>
<td>2.1 ± 0.5**</td>
</tr>
<tr>
<td>HSP72</td>
<td>1.0 ± 0.2</td>
<td>1.1 ± 0.2</td>
<td>45.7 ± 6.5**+</td>
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<tr>
<td></td>
<td></td>
<td>2.1 ± 0.5</td>
<td>45.1 ± 4.1**+</td>
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n = 8/group except fresh soles where n = 7. Statistics: Kruskal-Wallis followed by Wilcoxon post. *Compared with fresh soleus. +Heat compared with sham control (paired data). ++P < 0.001, *P < 0.01, +P < 0.05.

In contrast, group 2 animals that were not electrically stimulated at any time showed no measurable IL-6 protein in the baths in either control or heat. The data in Fig. 5 are normalized to the same bath size. The larger baths in either control or heat. The data in Fig. 5 are normalized to the

any time showed no measurable IL-6 protein in the baths in

(37°C). Force significantly declined by 10–20% in the hyperthermia group at twitch, 50, 80, and 150 Hz, when measured 1 h after return to 37°C. There was no measurable loss in force at any frequency over 2 h at 37°C in the sham controls (Fig. 5B).

In vivo experiments: comparison of IL-6, TNF-α, and HSP72 mRNA in soleus and plasma from normothermic vs. hyperthermic exposure. To determine if IL-6 or TNF-α cytokines are elevated in a physiologically intact model of hyperthermia, anesthetized mice underwent a heat protocol that gradually elevated core temperature over a period of several hours, as described previously (50). Soleus muscles were rapidly removed at the end of a 30 min recovery following attainment of the peak core temperature of 42.4°C. The mRNA levels were then compared with sham control muscles from mice exposed to the same protocol but kept at 37°C. IL-6, TNF-α, and HSP72 mRNA levels were evaluated, as shown in Fig. 6. The median IL-6 mRNA was elevated 5.4-fold (Fig. 6A), TNF-α was elevated 4.3-fold (Fig. 6B), and HSP72 was elevated 63-fold (Fig. 6C). Therefore, heat exposure, at levels known to induce heat stroke, stimulates IL-6 gene expression in skeletal muscle tissue of intact mice. However, unlike in isolated tissue or cells, TNF-α mRNA was also stimulated.

Plasma cytokines were also measured at the 30 min time point of recovery, as well as at 2 h after peak temperature was attained. These values were compared against samples from sham control animals at these time points (Fig. 7). Note the median plasma IL-6 was elevated to 477 pg/ml after 30 min of recovery and 1,314 pg/ml after 2 h of recovery, demonstrating a very robust circulating IL-6 response to heat. In contrast, TNF-α was elevated to 6.3 pg/ml at 30 min recovery, but was indistinguishable from sham controls at 2 h of recovery.

Mechanisms of heat-induced IL-6 mRNA message. Throughout the previous experiments, the individual responses of both HSP72 and IL-6 mRNA to heat exposure (in vivo, ex vivo, and in vitro) were highly correlated (Fig. 8). In contrast, in the tissue bath isolation experiments the mRNA responses of IL-6 and HSP72 were completely uncoupled (Table 1). This suggested the possibility that some independent component of IL-6 mRNA regulation may be tied to the same signaling pathways in heat that regulate HSP72, most likely via HSFs. To test this hypothesis, two different HSF inhibitors, quercetin (Sigma) and KNK437 (Calbiochem) (100 μM for each, dissolved in 0.1% DMSO vehicle), were given to C2C12 myotubes 6 h prior to heat exposure. Results were compared with control (37°C) treated with vehicle and control cells treated with the inhibitors. Results are shown in Fig. 9. As shown, quercetin treatment had a modest but insignificant effect on
heat-induced HSP72 expression and IL-6 expression compared with sham control 37°C cells also treated with quercetin but without heat. In contrast, KNK437 significantly inhibited both HSP72 mRNA and completely eliminated IL-6 mRNA expression in response to heat exposure when compared against sham cells that were unheated. Of note, these inhibitors had background influences on nonheated cells independent of heat, i.e., KNK437 increased background mRNA expression 4.2 ± 0.7-fold and quercetin increased expression 2 ± 0.5-fold. In contrast, both drugs significantly reduced background HSP72 mRNA expression in unheated/sham control cells (not shown).

Furthermore, we identified ATP/purinergic receptor activation and the NF-κB pathway as other candidate mechanistic pathways, possibly driving the upregulation of IL-6. In electrically stimulated C2C12 cells the most potent stimulus for IL-6 production is ATP/purinergic receptor activation (11). We blocked purinergic receptors with the inhibitor suramin (Sigma) (100 μM, dissolved in 0.9% saline). Additionally, the promoter region of the IL-6 gene has a NF-κB binding site and thus an important mediator for IL-6 gene activation (42). We used the cell-permeable inhibitor peptide SN50 to block this pathway (18 μM, dissolved in 0.9% saline). Interestingly, inhibition of ATP receptors and NF-κB translocation tended to further stimulate heat-induced IL-6 and HSP72 mRNA formation. Therefore, these experiments make it unlikely that either the purinergic receptor or the NF-κB pathways are important mechanisms for heat-induced IL-6 mRNA production.

**DISCUSSION**

The results of this study are consistent with the hypothesis that heat is a novel stimulus for IL-6 production in skeletal muscle. Moreover, the response is proportional to the extent of heat exposure, paralleling the expression of HSP72 mRNA at different temperatures across all models and showing sensitivity to pharmacological inhibition of HSP transcription in cell culture. Our results support a potential role of skeletal muscles as “heat stress sensors” that respond to hyperthermia exposure by producing elevated cytokines and chemokines, particularly IL-6, while suppressing other proinflammatory cytokines such as TNF-α. This pattern of response for muscle IL-6 mRNA is retained in conditions of heat stroke in the intact animal. However, in contrast, TNF-α is stimulated in these conditions, possibly from costimuli, such as endotoxin or catecholamines, known to be present in the circulation in heat stroke (1, 25). These observations may have important implications in understanding the integrated inflammatory responses observed during conditions of high fever, exertional hyperthermia, malignant hyperthermia, and heat stroke.

Possible mechanisms for heat-induced IL-6 formation. The idea that IL-6 expression may be another arm of the integrated cellular response to heat was first proposed by Hasselgren and colleagues, who showed that heat stress increases IL-6 mRNA in the intestinal mucosa of mice (71) and that when acute heat stress is applied to human enterocytes as a costimulus with IL-1β, IL-6 production is potentiated (52). HSF-1 has been proposed to have a role in partially opening the chromatin structure of the IL-6 promoter, which makes the promoter region more susceptible for activators or repressors to bind to it (27). Additionally, through the use of indirect activators of the heat shock transcription factors, IL-6 mRNA and protein expression were shown to be increased in the absence of hyperthermia, via HSF activation. Our data are entirely consistent with these findings and extend these studies to show that skeletal muscle may have an equally sensitive heat-induced IL-6 response.

Although signal transduction pathways for IL-6 gene regulation are extremely complex and include a wide variety of transcriptional regulators [as previously reviewed (53)], the simplest potential link between heat and IL-6 production would be via HSFs. When activated, HSFs form trimers, move to the nucleus and interact with heat shock elements (HSE) on the DNA, inducing a myriad of physiological responses, many of which are not directly related to expression of HSPs (21). Work in human intestinal epithelial cell lines has shown that the IL-6 gene promoter on the 5′-untranslated region contains several possible HSEs (55). We tested for these same sequences in mouse using a basic local alignment search tool (BLAST) search (National Center for Biotechnology BLAST) of the IL-6 gene on chromosome: 5;
NC_000071.5 (30339701..30346508) but were unable to locate the same HSE sequences identified in man within the 5' or 3' flanking regions (74). However, we did find one of the most potent HSE motifs [HSE3P, (74)] within the 2nd intron of the mouse IL-6 gene (numbering from 5'), beginning at nucleotide of 30340319: nTTCnnGAAnnTTCn [common form of the HSE3P type, HSE motif; (74)], tTTCtaGAAaaTTCa (sequence in the 2nd intron of IL-6 gene in the mouse). Such intronic regulatory sequences for HSF binding are common in many genes (21), and several have been identified as essential regulatory sites for transcriptional control of HSPs (13, 38). Therefore, there is strong potential for direct control of transcription of IL-6 via HSF-HSE interactions as originally proposed by Pritts et al. (55).

We further tested the link between HSF and IL-6 using common pharmacological blockers of HSF-HSE interactions, quercetin (a bioflavanoid) and KNK437 (a benzylidene lactam derivative) (26, 75). Only KNK437 significantly inhibited heat-induced IL-6 and HSP72 mRNA formation, whereas quercetin substantially blocked neither HSP72 nor IL-6 mRNA in this cell line. KNK437 has an advantage over quercetin in that it has no known antioxidant characteristics (75), and therefore it is a more specific test of the HSF-specific interactions on IL-6. Caution is warranted in interpretation of the KNK437 and quercetin experiments since they stimulated background IL-6 mRNA in nonheated cells, but in our experience treatment of myotubes with almost any solvent or mediator in modest concentrations elevates background IL-6. It appears to be an extremely sensitive regulatory system to external stimuli.

IL-6 and HSPs may share other common signaling pathways. For example, hyperthermia results in striking elevations in muscle ROS (77). ROS can stimulate both IL-6 (35) and HSP72 formation (24), possibly through the influence of damaged proteins on HSF induction. As an alternative hypothesis we considered the possibility that activation of ATP/purinergic receptors may be responsible for temperature-induced IL-6 mRNA stimulation. Activation of the ATP/purinergic receptors by electrical stimulation causes elevation in IL-6 mRNA in isolated C2C12 cells (11). Our rationale was that some phenotypes, such as erythrocytes, demonstrate a temperature-sensitive release of ATP in the ranges used in our study, which is believed to be an important vasodilatory signal in the skin.

**Fig. 7. Circulating cytokine levels: plasma IL-6 (A) and plasma TNF-α (B) taken from newly anesthetized control mice (fresh plasma), time-matched sham control mice 30 min after the protocol or 2 h after the protocol, and in animals exposed to a standardized HS protocol, taken to 42.4°C. Plasma samples were obtained at 30 min and 2 h after recovery at 37°C. Statistical analysis: nonparametric Newman-Keuls, followed by post hoc Wilcoxon; *P < 0.05, **P < 0.01. n.s., Not significant.**

**Fig. 8. Plot of IL-6 and HSP72 mRNA changes from intact skeletal muscle and C2C12 cells. CON myotubes (○), 40.5°C myotubes (□), 41°C myotubes (■), 42°C myotubes (△), in vivo soleus CON (×), in vivo soleus in HEAT (+), anesthetized soleus CON (+), anesthetized soleus HS (#). Best-fit slope = 0.3490, slope significantly different from zero P < 0.0001; r² = 0.6335.**

**Fig. 9. Pharmacological inhibition of HSF, ATP receptors and NF-κB signaling. A and B: responses of IL-6 and HSP72 mRNA to pharmacological inhibition of HSF. C and D: responses of IL-6 and HSP72 mRNA to pharmacological inhibition of ATP/purinergic receptors (suramin) and NF-κB (sn50). **P < 0.01. Results are from 2 samples each obtained in 4 separate experiments, i.e., 8 cultured wells for each sample.
during hyperthermia (31). However, blockade of the purinergic receptors did not depress but, rather, enhanced heat-induced IL-6 mRNA formation. The NF-κB pathway is another candidate pathway; it is a major signal by which IL-6 is transcribed in immune cells such as macrophages and lymphocytes (63), as well as in contracting rodent skeletal muscle (28). The cell-permeable inhibitor peptide SN50, known to inhibit translocation of the NF-κB active complex into the nucleus, was used to inhibit NF-κB activation. Similar to purinergic receptor blockade, IL-6 expression was not depressed, but enhanced.

Skeletal muscle is an ideal candidate to function as a heat sensor. It is a tissue of interest in exertional heat stress because of its capability to generate heat above core temperature. For example, during intense exercise in hot environments, skeletal muscle is as high as 41°C above core temperature, often reaching as high as 41°C in humans (20, 58) and as high as 44°C in rats (10). Since victims of severe heat stroke generally exhibit core temperatures ≥42°C, and severe fevers from infection or drug overdose can reach 41°C (34, 62), the range of temperatures at which we have observed significant elevations in muscle IL-6 mRNA (>41°C, Fig. 5) has broad physiological and pathophysiological relevance.

**IL-6 production in ex vivo soleus muscles.** In the isolated soleus muscles, overall effects of heat on IL-6 and TNF-α mRNA were nearly identical to those obtained in the isolated cell culture experiments. This suggests that the isolated C2C12 cell experiments are a relevant model for transcriptional regulation in response to heat. However, in every other way, the ex vivo soleus model presented a wide range of complexities and challenges in interpretation. First, we observed that the process of isolation and bath exposure had a large impact on both IL-6 (35- to 70-fold) and TNF-α mRNA (7.5-fold) expression, but no effect on HSP72 mRNA. Note that other muscle cytokine investigators have made similar observations with respect to cytokine upregulation in ex vivo muscle preparations (60). Based on the stability of the HSP72 mRNA, our results suggest that isolation had no influence on gene transcription or housekeeping genes per se, but rather induced a nonspecific proinflammatory stimulus that activated both TNF-α and IL-6 transcription or affected the stability of the mRNA. We eliminated three possibilities that might have caused this: endotoxin (by including polymyxin B in all buffers), asphyxia during isolation (by maintaining circulation until immediately before resection), and muscle stimulation (by eliminating stimulation during incubation). The only untested factors that we hypothesize could account for these persistent effects are: 1) mechanical manipulation of the muscle, as proposed previously (60), 2) high O2 partial pressures in the baths, which could induce oxidative stress and possibly stimulate ROS-sensitive inflammatory pathways such as via NF-κB (48), 3) low O2 partial pressures that would likely be present in the core of the soleus muscles when hung in the tissue baths (7), and 4) the use of a low volume tissue bath that could result in exaggerated upregulation through an autocrine mechanism (13). We feel that these stresses may be unavoidable in isolated whole muscle preparations, making it a difficult model to apply to questions regarding cytokine regulation.

The protein production in the isolated muscle experiments also yielded interesting and unexpected results. In the first experiment, group 1, the muscles were intermittently stimulated during incubation and the background IL-6 concentrations in the baths were extremely high in both control and heated muscle. We hypothesized that this was due to the overwhelming effect of isolation on IL-6 mRNA that simply washed out any independent effects of heat. In the second series, the muscles were not stimulated and though IL-6 mRNA was still greatly elevated due to isolation, there was no evidence of any IL-6 secretion under any condition. The only variable that we can identify that could account for these differences is the fact that group 1 underwent intermittent stimulations. We hypothesize that, in the intact muscle, transcriptional regulation and protein synthesis/protein secretion are not coupled to the same signaling systems. That is, mRNA and possibly protein production occur in response to one stimulus (heat, etc.), but secretion requires a second hit. There are ample precedents for this. For example, HSP72, another protein secreted in a variety of stress conditions, is ubiquitously upregulated in response hyperthermia, but only secreted upon a second stimulus characterized by high intracellular Ca2+ (29). Although little is known about the secretory pathways for IL-6 in skeletal muscle, in human mast cells, IL-6 is packaged in 40–80 nm vesicles, which must undergo some form of exocytosis (32). There are both Ca2+-dependent (39) and Ca2+-independent (32) secretory pathways for these vesicles. Therefore, we speculate that in the intact, unstimulated muscle, the lack of elevations in intracellular Ca2+ or perhaps other secretory mediators resulted in no net IL-6 secretion despite high levels of mRNA and possibly protein. So, why was there secretion in cultured myotubes? One possibility is that in many isolated cultured cells, hyperthermia induces marked elevations in intracellular Ca2+ (46). However, in adult differentiated skeletal muscle, the tight Ca2+ regulation afforded by the mature sarcoplasmic reticulum may have prevented hyperthermia-induced Ca2+ accumulation, thus requiring superimposed muscle stimulation to induce exocytosis of the IL-6 vesicles. Further experimentation will be necessary to explore these speculations.

**Regulation of IL-6 and TNF-α during in vivo heat stroke.** In the model of heat stroke used in this study, we demonstrated that there is a robust and very rapid elevation in circulating IL-6 and a small transient elevation in TNF-α. This pattern, seen in nearly all models of heat stroke (7, 8, 40, 57), resembles the responses of mRNA seen in the soleus muscles (Table 1) but differs from the responses seen in isolated muscle, where TNF-α was inhibited in heat. TNF-α is strongly stimulated by endotoxin, being a principle mediator of the innate immune response (6). Elevations in circulating endotoxin are commonly found during or following heat stroke, arising from intestinal barrier dysfunction (61). We hypothesize that the differing responses in muscle TNF-α expression in whole animal heat stroke, vs. isolated muscle during heat, reflect the overriding influence of other signals such as endotoxin or catecholamines that stimulate TNF-α (17, 37, 64). Since IL-6 is known to inhibit TNF-α expression in cell preparations (59, 73), it is also possible that the reduction of TNF-α mRNA seen in isolated muscle or cells could have occurred via an IL-6 autocrine inhibitory signaling pathway.

**Significance and Conclusions**

In this study we have identified heat as a stimulus for IL-6 expression in mouse skeletal muscle. However, if one considers the host of other stress stimuli which promote muscle IL-6 production that were previously mentioned (ROS, NO, Ca2+,
muscle isolation, endotoxin, glycogen depletion, catecholamines, ATP, exhausting exercise, etc.), it is logical to hypothesize that skeletal muscle may be poised to act as a whole organism “stress sensor” and that the response to heat facilitates other stress signals driving IL-6 expression. In this way it could play a role as a sentinel for detecting and integrating multiple stress signals and responding appropriately by producing endocrine proteins, such as IL-6, that are designed to protect the organism from harm. Alternatively, skeletal muscle’s sensitivity to local stress may be oriented toward initiating repair and remodeling mechanisms within the muscle microenvironment. We hypothesize that IL-6, which remains the most predominant myokine yet discovered, has a unique role to play. Recent evidence has shown that very low levels of IL-6 protect mammals from life-threatening conditions induced by severe hemorrhagic shock (3, 45, 47) and the fact that IL-6 knockout animals are more susceptible to heat stroke (41) provides theoretical support for this hypothesis.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


