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RESEARCH ARTICLE

Effect of heat stress on heat shock protein expression and hypertrophy-related signaling in the skeletal muscle of trained individuals

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Abstract

Muscle mass is balanced between hypertrophy and atrophy by cellular processes, including activation of the protein kinase B-mechanistic target of rapamycin (Akt-mTOR) signaling cascade. Stressors apart from exercise and nutrition, such as heat stress, can stimulate the heat shock protein A (HSPA) and C (HSPC) families alongside hypertrophic signaling factors and muscle growth. The effects of heat stress on HSP expression and Akt-mTOR activation in human skeletal muscle and their magnitude of activation compared with known hypertrophic stimuli are unclear. Here, we show a single session of whole body heat stress following resistance exercise increases the expression of HSPA and activation of the Akt-mTOR cascade in skeletal muscle compared with resistance exercise in a healthy, resistance-trained population. Heat stress alone may also exert similar effects, though the responses are notably variable and require further investigation. In addition, acute heat stress in C2C12 muscle cells enhanced myotube growth and myogenic fusion, albeit to a lesser degree than growth factor-mediated hypertrophy. Though the mechanisms by which heat stress stimulates hypertrophy-related signaling and the potential mechanistic role of HSPs remain unclear, these findings provide additional evidence implicating heat stress as a novel growth stimulus when combined with resistance exercise in human skeletal muscle and alone in isolated murine muscle cells. We believe these findings will help drive further applied and mechanistic investigation into how heat stress influences muscular hypertrophy and atrophy.

NEW & NOTEWORTHY We show that acute resistance exercise followed by whole body heat stress increases the expression of HSPA and increases activation of the Akt-mTOR cascade in a physically active and resistance-trained population.

heat shock proteins; heat stress; hypertrophy; resistance exercise

INTRODUCTION

Skeletal muscle is essential for basic motion, complex sport activities, and acts as a systemic regulator of homeostasis (1). With these adaptive capabilities, the development and maintenance of skeletal muscle mass is important not only for physical performance but also in aging and the retention of functional capabilities and health across the lifespan. Through a host of molecular events controlling myofibrillar protein synthesis and degradation, skeletal muscle is balanced between hypertrophy and atrophy (1, 2). Many factors contribute to and influence relative muscle anabolism and catabolism, including exercise, health status, and the progression of age (2). The protein kinase B (Akt)-mechanistic target of rapamycin (mTOR) pathway, including its downstream targets ribosomal S6 kinase β 1 (S6K1) and eukaryotic elongation initiation factor 4E-binding protein 1 (4E-BP1), are key control points for increasing myofibrillar protein synthesis and skeletal muscle hypertrophy in response to stimuli such as exercise (1). Furthermore, Akt-mTOR signaling inhibits relevant atrophy-related pathways that stimulate myofibrillar protein degradation via the ubiquitin-proteasome and autophagy lysosomal systems (3).

Interestingly, some evidence demonstrates that heat stress alone or in combination with resistance exercise can stimulate or enhance hypertrophic responses in skeletal muscle

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(4–7). There is emerging evidence from cellular-, animal-, and human-based investigations suggesting that heat stress of various forms can stimulate signaling related to muscle growth and result in skeletal muscle hypertrophy, protect against atrophy, and enhance recovery following muscle loss or damage (8). In addition, limited evidence indicates that the combination of resistance exercise and heat stress in humans, or exercise-like stimulation in cells and animals, can further promote the hypertrophic effects of heat stress (6, 7). Although evidence regarding these effects in humans and animals is equivocal, methodological differences including heating magnitude, type (environmental, heating pad and diathermy, hot water immersion, and microwave), duration (acute and chronic), the inclusion and type of resistance exercise (high load and low load), as well as timing of heating (pre, post, and concurrent) may account for some of the observed differences. Regardless, heat-induced responses in skeletal muscle including the expression, translation, and mediation via heat shock proteins (HSPs) may play a role in eliciting skeletal muscle adaptations (8). In fact, specific HSPs appear to interact with pertinent hypertrophy and atrophy-related signaling factors and may be implicit in the muscular responses discussed here (4, 7, 9, 10).

HSPs are ubiquitously expressed across mammalian tissues, including skeletal muscle, in response to various stressors including heat stress and exercise (11). Beyond their role in thermotolerance, these stress-inducible chaperones are implicated for their effects on cellular immunity, protein homeostasis, and the regulation of skeletal muscle mass across the lifespan in healthy and clinical populations (12). Of interest, specific HSPs of the HSP70 (HSPA) and HSP90 (HSPC) families (13, 14), HSP72 (HSPA1A), HSP70 (HSPA1B), HSP90-α (HSPC1), HSP90-α A2 (HSPC2), and HSP90-β (HSPC3), can interact with key hypertrophy and atrophy-related signaling factors with an emphasis on the Akt-mTOR and nuclear factor-κB (NF-κB) and Forkhead BOX O (FOXO) pathways (12, 15, 16). Reviewed elsewhere (8), these potential reciprocal effects could help explain observations that heat stress alone or combined with resistance exercise can enhance muscular hypertrophy or reduce atrophy. Nonetheless, these processes have yet to be robustly studied in skeletal muscle or in human populations. Moreover, there is no consensus regarding the ability of heat stress to meaningfully promote hypertrophy-related signaling compared with a known growth stimulus like resistance exercise.

The primary purpose of this investigation was to compare the individual effects of acute whole body heat stress, resistance exercise, and the combination of both interventions on thermoregulatory responses as well as heat shock and hypertrophy-related signaling responses in human skeletal muscle. We hypothesized that heat stress would increase core temperature to a greater degree than resistance exercise. Conversely, we proposed that resistance exercise would increase muscle temperature to a greater degree than heat stress. Finally, we hypothesized that the combination of both interventions would stimulate the greatest core and muscular responses. We next hypothesized that resistance exercise would increase Akt-mTOR signaling to a greater degree than heat stress alone. Conversely, we proposed that heat stress would induce greater heat shock signaling than resistance exercise. Finally, we hypothesized that the combination of resistance exercise and heat stress would result in greater Akt-mTOR and heat shock signaling to a greater degree than either intervention alone. Coinciding with our human investigation, we also examined the effect of acute heat stress on the development of cultured mouse myotubes subjected to hypertrophy and atrophy-stimulating conditions. We proposed that heat stress would increase myotube hypertrophy and myonuclear fusion index compared with controls but to a lesser degree than hypertrophy conditions. In addition, we proposed that atrophy conditions would reduce growth and development compared with controls, heat stress, and hypertrophy conditions. A secondary purpose of this investigation was to examine the whole body and muscle thermoregulatory responses to each intervention in our human investigation.

METHODS

Participants

All study protocols were approved by the University of New Mexico (UNM) Institutional Review Board and conducted in accordance with the Declaration of Helsinki. Participants were recruited by word of mouth from the UNM and surrounding Albuquerque area. All study activities were completed in the UNM Exercise Physiology Laboratories including the dedicated gym space, environmental heat chamber, and private rooms. Participation in this study was voluntary with all participants providing verbal and written informed consent. After consent was obtained, volunteers completed health history and physical activity questionnaires to determine health and fitness status (Table 1). All participants (males $n = 5$, females $n = 3$, 25–32 yr old) were deemed healthy, did not participate in regular heat training or exposure, and reported participation in at least 150 min of moderate-intensity physical activity plus two or more days of structured resistance exercise per week and lived in the Albuquerque area (~1,600 m) for a minimum of 6 mo. After consent and health screening, participants visited the laboratory to complete baseline testing followed by three experimental trials including resistance exercise (RE), whole body passive heat exposure (HS), and RE followed by HS (REH) as detailed in Fig. 1. Experimental trials were completed in a randomized and counterbalanced fashion with 14–21 days of washout between conditions and were conducted during the Fall and Spring.

Resistance Exercise, Heat Stress, and Resistance Exercise-Heat Stress Protocols

During baseline testing, participant demographics (age, sex, height, weight, and body fat %), estimated 1 repetition maximum (RM) for seated leg press and knee flexion, as well as 10 RM for seated knee extension and standing calf raise, were determined. Sex-specific three-site skinfolds and density equations for males (chest, abdomen, and thigh) and females (triceps, iliac crest, and thigh) were used to estimate body fat percentage via the Siri equation (17). RMs were determined following a 5-min warm-up on a cycle ergometer at a self-selected pace by progressively increasing weight lifted until no more than 5 or 10 repetitions could be completed for leg press and knee flexion and knee extension and

Table 1. *Participant demographics*

Sex	Age	Height, cm	Weight, kg*	Body Fat, %*	1 RM Leg Press, kg*
Males ($n = 5$)	25 ± 3.3	175 ± 9.3	71.8 ± 9.0	9.4 ± 4.3	590.2 ± 78.0
Females ($n = 3$)	32 ± 5.6	163 ± 9.4	59.8 ± 3.5	16.9 ± 4.2	415.7 ± 60.5

Participant descriptive data for physically active (≥150 min moderate activity per week), resistance trained (≥2 days per week ≥6 mo) males ($n = 5$) and females ($n = 3$). All data presented as group means ± SD. Sex differences analyzed with one-tailed t tests for unequal variance. *Significant difference between groups for column category. Age ($P = 0.07$), height ($P = 0.07$), weight ($P = 0.02$), body fat % ($P = 0.03$), 1 RM leg press ($P < 0.01$). RM, repetition maximum.

calf raise, respectively, with 3 min of rest between sets. Participants arrived for all experimental trials following an overnight fast having refrained from vigorous exercise and alcohol consumption for at least 24 h, and caffeine for 4 h. Before the heat conditions, participants were determined to be hydrated via urine specific gravity <1.020. RE was preceded by a cycling warm-up and then a set of 10–15 repetitions for leg press and knee flexion at 50% of 1 RM. In total, resistance exercise lasted for ~60–70 min including five sets of 8–12 repetitions of leg press and knee flexion at 75% of 1 RM followed by three sets of 10 repetitions of knee extension and calf raises at 100% of 10 RM. Each paired exercise (leg press and knee flexion, knee extension, and calf raise) was alternated with 3 min of rest between sets. The number of repetitions, rating of perceived exertion (RPE), and heart rate (HR) were measured at the end of each set, while core temperature was measured pre- and posttrial via thermistor (ICU Medical Level 1, Smiths Medical, Minneapolis, MN) inserted ~10 cm past the anal sphincter. HS included 60 total min of passive, seated heat exposure in an isolated environmental heat chamber maintained at 55–60°C and 20–30% relative humidity. To assist in participant comfort, participants were given 5-min breaks every 20 min. During these breaks, participants were allowed 1 min in a temperate environment (~20–22°C) and the remaining 4 min in the environmental antechamber (~10°C cooler than the isolated chamber). This protocol was adopted following pilot testing that revealed temperatures above 50°C at 20–30% relative humidity were necessary to robustly increase core and muscle temperatures. Every 10 min, RPE, HR, thermal sensation (subjective scale ranging from 0 = very cold to 8 = very hot), core temperature, as well as dry and wet bulb temperatures were recorded. The REH trial included the RE protocols immediately followed by the HS protocols as described previously in methods. Participants were allowed room temperature water ad libitum throughout all trials. Following each experimental condition, participants rested in a temperate environment between the 30-min and 3-h posttrial biopsies.

Muscle Biopsy and Temperature Protocols

For each experimental trial, muscle tissue samples were collected from the m. vastus lateralis muscle pre-, 30-min post-, and 3-h posttrial. The limb was sanitized with sterile alcohol swabs, antiseptic (iodine), followed by superficial and deep injection of ~3–5 mL of local anesthesia (2% lidocaine). After verification of superficial numbness, the superficial fascia of the muscle was pierced with a 14-gauge hollow needle. A 14-gauge biopsy needle (Argon Medical Devices,

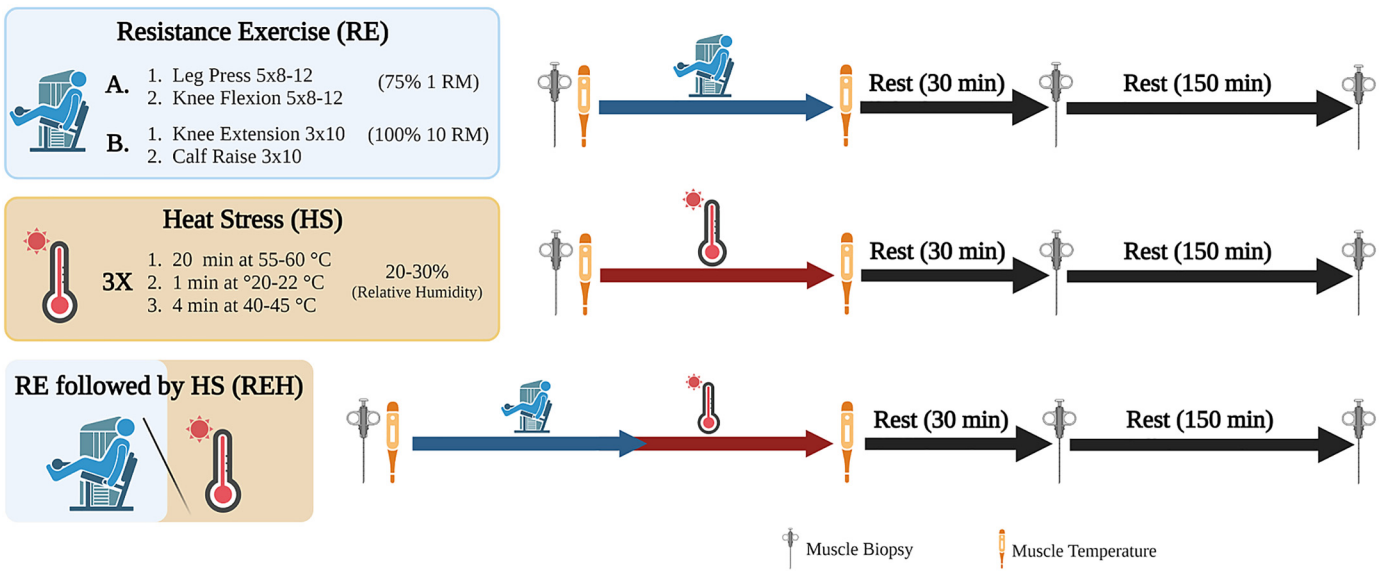

Figure 1. Timeline for experimental trials including resistance exercise (RE), whole body heat stress (HS), and RE followed by HS (REH). RE, HS, and REH were completed in a randomized and counterbalanced fashion with 14–21 days of washout between trials. Trials occurred following consent, health screening, and baseline testing including demographic as well as 10 rep maximum (RM) and estimated 1 RM strength testing. Three minutes of rest was given between all RE exercise sets. After 20 min of HS at 55–60°C (20–30% relative humidity), participants took comfort breaks for 1 min at 20–22°C and 4 min at 40–44°C before returning to 55–60°C. Muscle temperature was measured pretrial and immediately posttrial for each condition. Skeletal muscle biopsies were taken pre-, 30 min post-, and 3 h posttrial from the v. lateralis for each condition.

Frisco, TX) was inserted via the pilot incision twice to obtain ~10–20 mg of total tissue. Incision sites were cleaned and wrapped in sterile bandaging. Tissue samples were cleaned of debris, washed in cold phosphate-buffered saline (PBS), flash frozen in liquid nitrogen, and stored at −80°C for Western blot analysis. Muscle temperature was measured pretrial and immediately posttrial for each experimental condition. Muscle temperature readings were performed in the same site as the baseline biopsy for all trials. During the pretrial muscle biopsy, a sterile implantable temperature probe (IT-18, Physitemp Instruments, Clifton, NJ) was inserted past the superficial fascia (~2–3 cm) through the hollow pilot needle before collecting tissues. Muscle temperature was recorded from a calibrated microprobe thermometer (BAT-12, Physitemp Instruments) with the pilot needle removed. Posttrial muscle temperature was measured within 5 min of trial cessation using the same methods excluding tissue collection.

Western Blot Protocols

Muscle tissue was lysed with ~300 μL of ice-cold lysis buffer (General Cell Lysis Buffer, Millipore Sigma, St. Louis, MO) containing protease and phosphatase inhibitors plus EDTA (Halt, Thermo Fisher Scientific, Waltham, MA) for 60–120 s in disposable BeadBug tubes with 1.5-mm zirconium beads (Millipore Sigma) at 3,000 RPMs for 1–2 rounds of 60 s until homogenized (Beadbug 3, Benchmark Scientific, Sayreville, NJ). After the addition of 4X Laemmli buffer (Bio-Rad Laboratories, Hercules, CA) with 5% β-mercaptoethanol, samples were incubated at 95°C for 10 min. Total protein (20 μg, Pierce BCA, Thermo Fisher Scientific) was separated by electrophoresis on 4–20% polyacrylamide gels, and 1x Tris/glycine/SDS running buffer (Bio-Rad Laboratories), and then transferred using 1x Tris/glycine transfer buffer with 10% methanol (Bio-Rad Laboratories) to 0.45-μm PVDF membranes (Thermo Fisher Scientific) via Trans-blot Turbo Transfer using the SD protocol (Bio-Rad Laboratories). Membranes were blocked for 90 min in 5% bovine serum albumin (BSA) or dry milk in Tris-buffered saline plus 0.05% Tween 20 buffer solution (TBST), cut according to molecular weight based on dual-color standards (Bio-Rad Laboratories), washed with TBST, and incubated overnight at 4°C with primary antibodies in BSA-TBST; phospho-Akt (Ser473, no. 9271, AB_329825), Akt (no. 9272, AB_329827), phospho-mTOR (Ser2448, no. 2971, AB_330970), mTOR (no.2972, AB_330978), phospho-S6 kinase 1 (Thr389, no.9205, AB_330944), S6 kinase 1 (49D7, no.2708, AB_390722), phospho-4E-BP1 (Thr37/46, no.2855, AB_560835), 4E-BP1 (53H11, no.9644, AB_2097841), HSPA (no.4872, AB_2279841), HSPC (no.4874, AB_2121214) (all antibodies purchased from Cell Signaling Technology, Danvers, MA); diluted 1:1,000 per manufacturer recommendations. Membranes were washed with TBST and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) diluted 1:1,000 (Akt-mTOR) or 1:2,000 (HSPs) for 1 h at room temperature. Membranes were covered in luminol reagent (100 mM Tris·HCl pH 8.8, 1.25 mM luminol-DMSO, 2 mM 4IBPA-DMSO, 5.3 mM H_2O_2) (18) for 3 min and imaged (Cheick Touch Imaging System, Bio-Rad Laboratories). Image Lab software was used to quantify protein expression (Bio-Rad Laboratories). After initial phospho-protein probing, membranes were stripped in mild stripping buffer (200 mM glycine, 3.5 mM SDS, 1% Tween-20, pH 2.2) twice for 5 min on a rocker followed by washing in PBS, TBST, and then 90 min blocking in milk-TBST. Membranes were then incubated for corresponding total Akt-mTOR proteins as described above. All time points for individual participants were performed on the same gels. All proteins were standardized to total protein staining (Ponceau S Stain, Cell Signaling Technology) and expressed as peak, max, or min where appropriate, and a ratio of phospho to total protein where applicable, in densiometric units with the pretrial time point for each condition serving as baseline. If total protein, and thus phosphorylation, status for any participant was undetectable, data were not included. This was true for one participant considering Akt, and three participants for 4E-BP1.

Cell Culture Experiments

Cell culture.

C2C12 myoblasts (American Type Culture Collection, passages 3–7) were used for all cellular protocols, initially seeded in 150-mm culture plates (~5×10^6) until ~50–70% confluency then passed 1:6 into 35-mm, six-well culture plates (Corning) in Dulbecco's modified Eagle's medium (DMEM) media (Sigma Aldrich) with 4.5 $g \cdot mL^{-1}$ glucose, L-glutamine, sodium pyruvate, 1% penicillin-streptomycin, and 10% fetal bovine serum (Sigma Aldrich). Myoblasts were cultured to ~90% confluence under standard conditions (37°C and 5% CO_2) with media changes occurring every 24–36 h for ~6 days. Myotubes were differentiated in DMEM containing 2% horse serum (Sigma Aldrich) in place of FBS and changed every 24 h for ~5 days until myotubes developed.

Cell treatment protocols.

Experimental treatments included control, heat stress, growth media, and rapamycin treatments. Control replicates ($n = 9$) were maintained in standard conditions throughout. Heat stress ($n = 8$) included 60 min at 40°C and 5% CO_2 followed by return to standard conditions in differentiation medium. Growth media treatment ($n = 9$) included exposure to growth medium under standard conditions for 48 h (19). Rapamycin treatment ($n = 9$) included exposure to rapamycin (Thermo Fisher Scientific)-treated differentiation medium (164 nM, 0.25% DMSO) (20) for 48 h. A single media change occurred for all conditions at 24 h.

MHC staining and analysis protocols.

Forty-eight hours after experimental treatments, myotubes were aspirated of media, rinsed in PBS, and fixed in 4% paraformaldehyde (PFA) on a plate rocker for 5 min. Myotubes were then permeabilized with 0.3% Triton X-100 in PBS for 5 min, followed by another 5 min of fixation in PFA. Myotubes were rinsed twice in PBS with 0.01% Tween-20 then blocked in 3% BSA in PBS on a plate rocker for 60 min. Myotubes were incubated in 3% BSA containing primary MHC antibodies (MF20, DSHB) at 1.5 μg/mL on a plate rocker overnight. After three rinses in PBS, myotubes were incubated in PBS with secondary antibody diluted 1:500 (Alexafluor 488, Thermo Fisher Scientific) on a plate rocker for 60 min. Myotubes were washed three times with PBS, incubated for

10 min in 5 μM DAPI, and then rinsed three times. Three images were taken per well for each replicate with randomly selected locality using an inverted microscope in a 20 × 20 field (Olympus CKX53, Life Science Solutions, San Diego, CA). Images were analyzed using MyoCount software (v1.3.1) (21) via MATLAB Runtime (v9.4, R2018a, MathWorks, Natick, MA) for myotube area (% of area occupied by functional myotubes), total number of nuclei, and nuclear fusion index (number of nuclei within functional myotube/total nuclei). Functional myotubes were identified as those containing at least three nuclei. Images were averaged as a single measure for each condition replicate. One well for the heat stress ($n = 8$) did not reach confluency and was not included in further analysis.

Statistical analyses.

Participant recruitment numbers were based on a priori effect size (0.8) power analysis in G*Power (22) from an investigation of whole body heat stress on muscle temperature, core temperature, and protein responses (4). As six participants, not considering sex, were required to reach estimated power, we set a total recruitment goal of 8. The distribution of all data was examined visually with Q-Q plots to assess statistical assumptions and normality. Baseline demographic differences between sexes were examined with one-way *t* test for unequal variance. Perceptual data including thermal sensation and RPE, as well as environmental data including dry and wet bulb temperatures, were analyzed with repeated-measures *t* tests for equal variance. Because of technical difficulty or participant discomfort, three preresistance exercise core temperature measures for REH as well as two and one muscle temperature measures for REH and HS trials were not recorded, respectively. Data were examined for normality, skewness, kurtosis, and analyzed using repeated-measures ANOVAs with Geisser-Greenhouse epsilon corrections, as well as Holm–Sidak multiple comparisons where relevant. As the Holm–Sidak method provides greater power across multiple comparisons but cannot compute confidence intervals, we have simply provided the mean difference ± standard error of the mean for post hoc comparisons where relevant. If data points were missing, linear mixed-effects models were used as they are robust to missing data. Our Western blot data did not conform to tests of normality and therefore were log transformed (Base 10), checked for approximate normality, and analyzed using repeated-measures or mixed-effect ANOVAs. For all conditions, protein expression data were first analyzed considering their absolute expression at both the 30-min and 3-h time points. In addition, results were then analyzed using the individual peak value, minimum or maximum as appropriate, and determined at either the 30-min or 3-h time point to account for individual variability in signaling responses (4). All time points irrespective of peaks are additionally provided. The magnitude of change for main effects was examined using partial omega squared (ω_p^2) for two-way and R^2 for one-way ANOVAs. Both ω_p^2 and R^2 indicate the proportion of total variation accounted for by the interaction between variables. As a general rule of thumb, a small effect falls between $\leq$0.02 to $<$0.13, a medium effect $\geq$0.13 to $<$0.26, and a large effect $\geq$0.26. Statistical significance for all analyses was set at $P \leq 0.05$ and was performed in R (v 4.2.0, R Foundation for Statistical Computing, Vienna, Austria) via RStudio (v 2022.02.), GraphPad Prism, (v 10.0.1GraphPad Software, La Jolla, CA), or Excel (v16.0, Microsoft Corp., Redmond, WA). Undiscussed or insignificant protein and physiological responses for all time points and sex differences are available as supplementary data (Supplemental Tables S1 and S2).

RESULTS

Thermoregulatory Responses

Core temperature increased from baseline [$F(1,18) = 85.19$, $P < 0.01$) for RE (Δ0.53 ± 0.35°C, $P < 0.01$], HS (Δ1.30 ± 0.63°C, $P < 0.01$), and REH (Δ1.05 ± 0.42°C, $P < 0.01$) (Fig. 2*A*). A time × condition effect [$F(2,18) = 5.44$, $P = 0.01$] indicated baseline core temperature was not different between any condition ($P = 0.99$) but posttrial core temperature following RE was lower than HS ($P < 0.01$) and REH ($P < 0.01$) while posttrial core temperature for HS and REH was not different ($P = 0.86$) (Fig. 2*A*). Time course analysis also demonstrated that core temperature increased from baseline [$F(1.31,9.22) = 31.70$, $P < 0.01$] but was not different between conditions [$F(1,7) = 1.09$, $P = 0.33$], despite REH displaying greater average core temperature compared with HS heating sessions. Accordingly, regardless of a significant time × interaction effect, core temperature during RE and REH heating sessions was not significantly different at any specific time point (Fig. 2*C*). Muscle temperature increased from baseline [$F(1,38) = 102.4$, $P < 0.01$] for RE (Δ2.04 ± 1.30°C, $P < 0.01$), HS (Δ2.49 ± 1.58°C, $P < 0.01$), and REH (Δ3.25 ± 1.14°C, $P < 0.01$). There was not a significant time-by-condition effect for muscle temperature [$F(2,18) = 2.43$, $P = 0.16$] (Fig. 2*B*). Time course analysis revealed that HR increased across time [$F(1.81,12.69) = 15.14$, $P < 0.01$] and was different between conditions [$F(1,7) = 8.23$, $P = 0.02$] comparing RE (113 ± 18 beats/min) and REH (101 ± 11 beats/min) heating sessions. Accordingly, average HR was significantly greater during REH compared with HS at the zero ($P < 0.01$) and the 10-min time point ($P = 0.02$) (Fig. 2*D*). During RE and REH exercise sessions, HR increased across time [$F(2.63,18.43) = 14.69$, $P < 0.01$] but was not different between conditions [$F(1, 7) = 0.26$, $P = 0.62$] despite RE displaying greater average HR (132 ± 22 beats/min) compared with REH (123 ± 23 beats/min) (Fig. 2*E*). Average RPE was not different between RE (14.8 ± 1.0) and REH (14.6 ± 1.1) exercise sessions ($P = 0.49$) or REH (11.5 ± 1.8) and HS (10.4 ± 2.5) heating sessions ($P = 0.17$). Average thermal sensation was not different when comparing HS (6.7 ± 0.47) and REH (6.8 ± 0.45) heating sessions ($P = 0.75$). Average dry (58.0 ± 1.3 vs. 58.1 ± 2.3°C, $P = 0.65$) and wet (35.4 ± 2.9 vs. 35.9 ± 1.1°C, $P = 0.84$) bulb temperatures were not different comparing HS and REH heating sessions.

Protein Expression

Herein, HSP responses are presented as the change in protein expression from baseline as means ± SE. Results are presented first as absolute expression at the 30-min and 3-h time points and second, considering the grouped peak expression value as described in our methods and by others (4). Both absolute and peak responses are displayed alongside a representative image including all proteins at the 30-

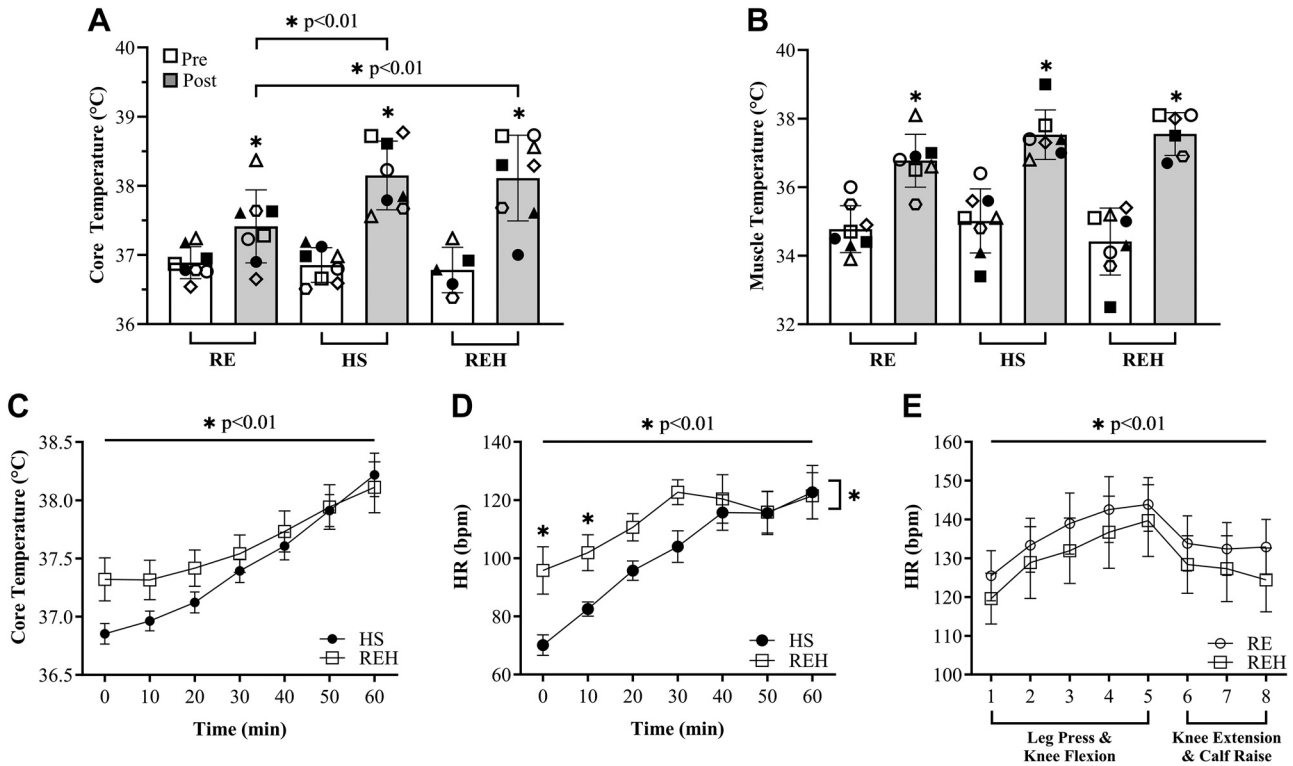

Figure 2. Thermoregulatory responses to resistance exercise (RE), heat stress (HS), and RE followed by HS (REH). *A*: pre- and posttrial core temperature values for all conditions. *B*: pre- and posttrial muscle temperature values for RE and HS. Each plotted shape represents the same participant throughout conditions. Group average pre- values represented by white bars and group average post values by gray bars with standard deviation error bars. Each plotted shape and color represents the same participant or condition as indicated. Females ($n = 3$) are represented by filled black shapes and males ($n = 5$) by white shapes. *Significant difference compared with baseline, while asterisk above brackets indicates significant difference between average values as indicated. Analyzed via repeated-measures or mixed-effects ANOVA. *A*: RE ($P < 0.01$), HS ($P < 0.01$); *B*: RE ($P < 0.01$), HS ($P < 0.01$). *B*: REH ($P < 0.01$), HS ($P < 0.01$), and REH ($P < 0.01$). *C*: core temperature across time for HS and REH heat sessions. *D*: heart rate across time for HS and REH heat sessions. *E*: HR across time for RE and REH exercise sessions. Each plotted point represents the group average for that time point with standard deviation error bars. Asterisk over bar indicates significant effect of time and difference from baseline, asterisk above individual points a significant difference between groups per indicated time point, and next to brackets a significant difference between group averages for conditions. Time course responses are analyzed via repeated-measures or mixed-effects ANOVAs. *C*: RE vs. REH ($P = 0.33$). *D*: HS vs. REH ($P = 0.02$), HS vs. REH at 0 min ($P < 0.01$) and 10 min ($P = 0.02$). *E*: RE vs. REH ($P = 0.62$).

min and 3-h time points and total protein staining (Fig. 3*E*). Absolute HSPA expression changed across time [$F(1.8,38.7) = 9.1$, $P < 0.01$, $\omega_p^2 = 0.26$] but not condition ($P = 0.21$, $\omega_p^2 = 0.05$), and there was not a significant time × condition effect ($P = 0.11$, $\omega_p^2 = 0.07$). Compared with baseline, HSPA expression was increased for REH at the 3-h time point [fold change (FC) = 0.17 ± 0.06, $P = 0.04$] only (Fig. 3*A*). Peak HSPA expression changed across time [$F(1,21) = 20.2$, $P < 0.01$, $\omega_p^2 = 0.45$] and was increased from baseline following HS (FC = 0.27 ± 0.11, $P = 0.04$) and REH (FC = 0.40 ± 0.20, $P < 0.01$) but not RE ($P = 0.13$) (Fig. 3*C*). There was not a significant effect of condition ($P = 0.32$, $\omega_p^2 = 0.01$) or time × condition ($P = 0.32$, $\omega_p^2 = 0.01$) for peak HSPA expression. Absolute HSPC expression changed across time [$F(1.6,35.4) = 4.0$, $P = 0.03$, $\omega_p^2 = 0.04$] but was not different from baseline for RE, HS, or REH at any time point (Fig. 3*B*). There was not a significant effect of condition ($P = 0.58$, $\omega_p^2 = 0.11$) or time × condition ($P = 0.80$, $\omega_p^2 < 0.01$) for absolute HSPC expression. Peak HSPC expression changed across time [$F(1,21) = 6.7$, $P = 0.01$, $\omega_p^2 = 0.19$] and was decreased from baseline following HS (FC = −0.50 ± 0.20, $P = 0.03$) but not REH ($P = 0.83$) or RE ($P = 0.12$) (Fig. 3*D*). There was not a significant effect of condition ($P = 12$, $\omega_p^2 = 0.09$) or time × condition ($P = 0.12$, $\omega_p^2 = 0.09$) for peak HSPC expression.

Protein Phosphorylation Events

Akt-mTOR phosphorylation events are presented as the change in ratio of phosphorylated to total protein compared with baseline as means ± SE. Results are presented first as absolute phosphorylation ratio for the 30-min and 3-h time points and second, considering the grouped peak expression value as described previously. Both absolute and peak phosphorylation responses are displayed in Fig. 4 alongside a representative image including all proteins at the 30-min and 3-h time points and total protein staining (Fig. 4*I*). Absolute Akt protein phosphorylation ($n = 7$) did not change across time ($P = 0.43$, $\omega_p^2 < 0.01$) or condition ($P = 0.09$, $\omega_p^2 = 0.14$), and there was not a significant time × condition interaction ($P = 0.10$, $\omega_p^2 = 0.09$) (Fig. 4*A*). Peak Akt responses changed across time [$F(1,18) = 4.4$, $P = 0.04$, $\omega_p^2 = 0.14$] but were not different from baseline for RE ($P = 0.18$) or REH ($P = 0.66$) despite a trend to be increased for HS (FC = 0.42 ± 0.17, $P = 0.08$) (Fig. 4*E*). There was not a significant effect of condition ($P = 0.14$, $\omega_p^2 = 0.09$) or time × condition ($P = 0.14$, $\omega_p^2 = 0.09$) for peak Akt responses. Absolute mTOR protein

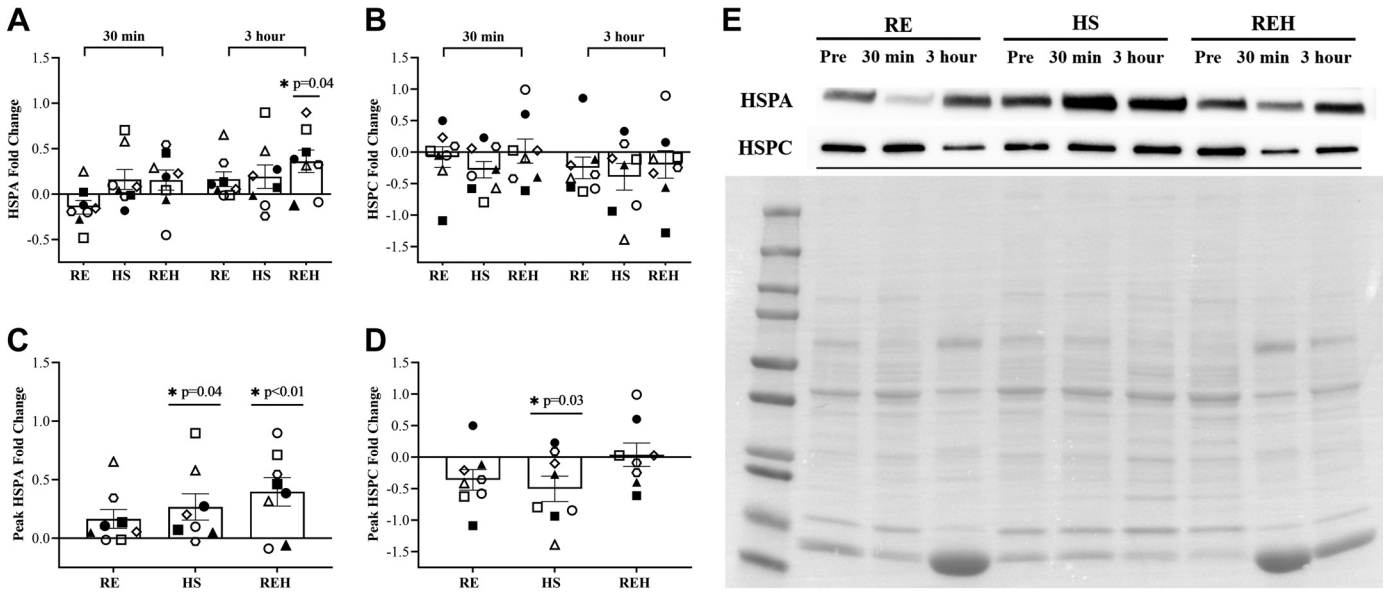

Figure 3. HSP protein expression following resistance exercise (RE), heat stress (HS), and RE followed by HS (REH). *A* and *B*: absolute expression at both 30 min and 3-h time points compared with baseline HSPA and HSPC. *C* and *D*: peak expression at either 30 min or 3 h posttrial compared with baseline for HSPA ($n = 8$) and HSPC ($n = 8$). Each plotted shape and color represents the same participant or condition as indicated. Females ($n = 3$) are represented by filled black shapes and males ($n = 5$) by white shapes. Bars represent mean fold change ± SE of the mean error bars. All proteins corrected to total (Ponceau staining) and log transformed (base 10). *Significant difference compared with baseline for indicated protein. *E*: representative image of protein expression for protein targets at baseline (pre), 30 min post, and 3 h posttrial for RE, HS, and REH and total protein stain. Analyzed via repeated-measures ANOVAs. HSPA, heat shock protein A; HSPC, heat shock protein C.

phosphorylation ($n = 8$) changed across time [$F(1.64,34.51) = 4.28$, $P = 0.02$, $\omega_p^2 = 0.13$] but not condition ($P = 0.29$, $\omega_p^2 = 0.12$) or time × condition ($P = 0.71$, $\omega_p^2 < 0.01$). Compared with baseline, mTOR phosphorylation was increased for REH at 30 min (FC = 0.24 ± 0.08, $P = 0.02$) only while HS demonstrated a trend toward significance at the 3-h time point (FC = 0.21 ± 0.07, $P = 0.06$) (Fig. 4*B*). Peak mTOR responses changed across time [$F(1,21) = 24.5$, $P < 0.01$, $\omega_p^2 = 0.50$] and increased from baseline following HS (FC = 0.49 ± 0.16, $P = 0.02$) and REH (FC = 0.66 ± 0.44, $P < 0.01$) but not RE ($P = 0.28$) (Fig. 4*F*). There was not a significant effect of condition ($P = 0.29$, $\omega_p^2 = 0.02$) or time × condition ($P = 0.29$, $\omega^2 = 0.02$) for peak mTOR responses. Absolute S6K1 protein phosphorylation ($n = 8$) did not change across time ($P = 0.25$, $\omega_p^2 = 0.02$), condition ($P = 0.39$, $\omega_p^2 < 0.01$), or time × condition ($P = 0.61$, $\omega_p^2 < 0.01$) (Fig. 4*C*). Peak S6K1 protein phosphorylation changed across time [$F(1,21) = 6.9$, $P = 0.01$, $\omega_p^2 = 0.20$] and was increased from baseline following HS (FC = 0.50 ± 0.37, $P = 0.05$) but not RE ($P = 0.18$) or REH ($P = 0.80$) (Fig. 4*G*). There was not a significant effect of condition ($P = 0.26$, $\omega_p^2 = 0.03$) or time × condition ($P = 0.26$, $\omega^2 = 0.03$) for peak S6K1 responses. Absolute 4E-BP1 protein phosphorylation ($n = 5$) did not change across time ($P = 0.20$, $\omega_p^2 = 0.04$), condition ($P = 0.62$, $\omega_p^2 < 0.01$), or time × condition ($P = 0.42$, $\omega_p^2 < 0.01$) (Fig. 4*D*). Peak 4E-BP1 responses changed across time [$F(1,12) = 9.0$, $P = 0.01$, $\omega_p^2 = 0.36$] but were not different from baseline for RE ($P = 0.41$), or REH ($P = 0.15$), despite a trend to be reduced for HS (FC = −0.22 ± 0.12, $P = 0.09$) (Fig. 4*H*). There was not a significant effect of condition ($P = 53$, $\omega_p^2 < 0.01$) or time × condition for peak S6K1 ($P = 0.53$, $\omega^2 < 0.01$).

Cell Culture Results

Myotube area was influenced by treatment [$F(3,31) = 26.36$, $P < 0.01$, $R^2 = 0.71$] and all treatments were different from control conditions (Fig. 5*A*). Myotube area was lower following rapamycin compared with control treatment ($P = 0.02$) with average area of 35.3 ± 3.7 and 39.6 ± 3.8%, respectively. Myotube area was greater following heat stress compared with control ($P = 0.03$) and rapamycin treatments ($P < 0.01$) with average area of 43.0 ± 2.43%. Myotube area was greater following growth media compared with control, rapamycin, and heat stress treatments ($P < 0.01$), with average area of 48.7 ± 2.8%. Fusion index was also influenced by treatment [$F(3,31) = 11.57$, $P < 0.01$, $R^2 = 0.52$] (Fig. 5*B*). Fusion index was unchanged following rapamycin compared with control treatment ($P = 0.44$) with average values of 0.37 ± 0.04 and 0.39 ± 0.03, respectively. Fusion index was greater following heat stress compared with rapamycin ($P < 0.01$) and control treatment ($P = 0.02$) with average values of 0.45 ± 0.02. Fusion index was greater following growth media treatment compared with rapamycin ($P < 0.01$) and control treatments ($P < 0.01$) with an average value of 0.46 ± 0.03. Fusion index was not different when comparing heat stress and growth media treatment ($P = 0.65$). Total number of nuclei was not different between any condition [$F(3,31) = 1.90$, $P = 0.14$, $R^2 = 0.15$].

DISCUSSION

The primary purpose of this investigation was to compare the individual effects of acute RE, whole body HS, and REH on thermoregulatory responses as well as skeletal muscle

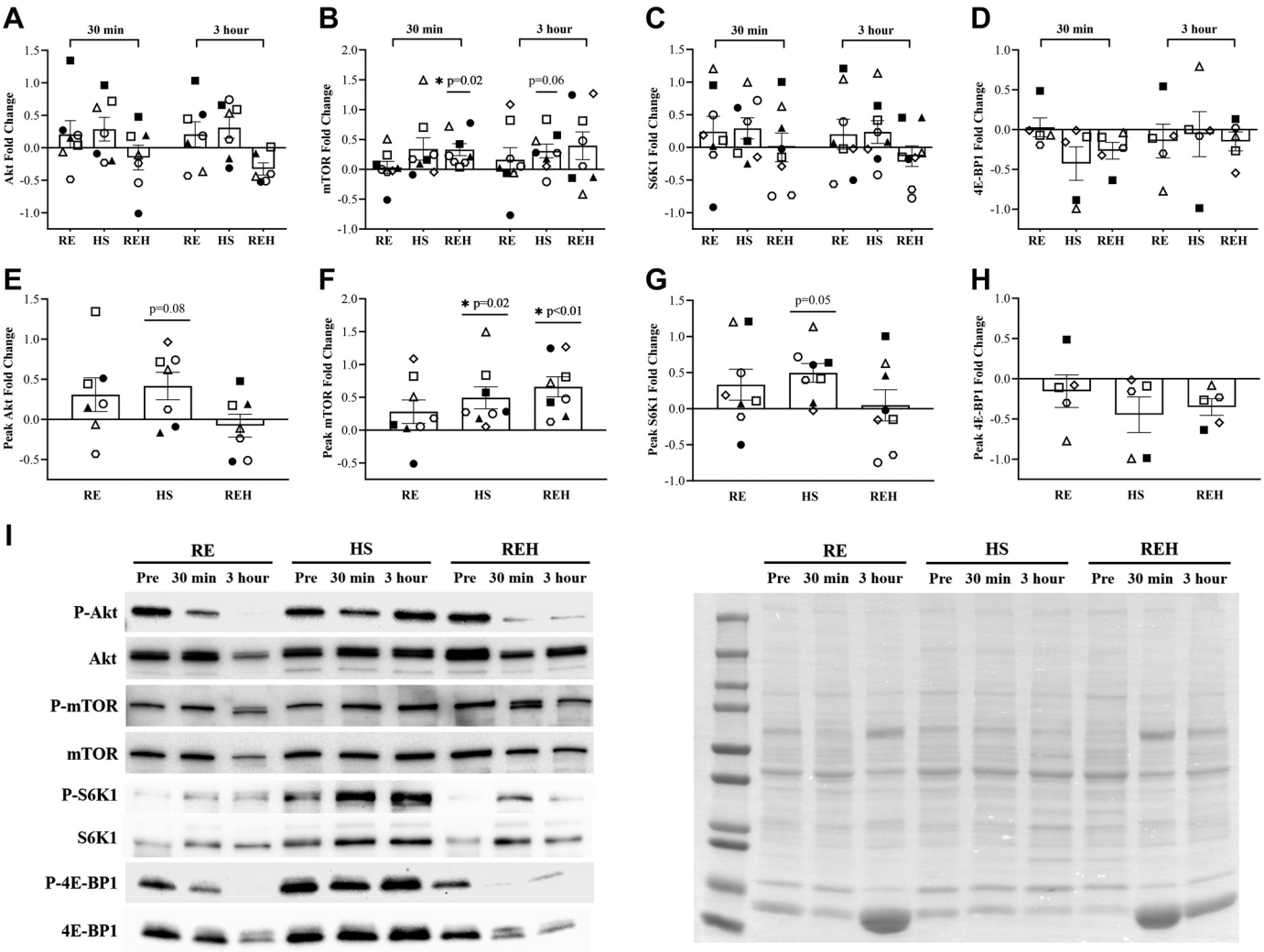

Figure 4. Akt-mTOR protein phosphorylation following resistance exercise (RE), heat stress (HS), and RE followed by HS (REH). *A–D*: absolute phosphorylation at both 30-min and 3-h time points compared with baseline for Akt, mTOR, S6K1, and 4E-BP1. *E–H*: peak phosphorylation occurring at either 30 min or 3 h posttrial compared with baseline for Akt ($n = 7$), mTOR ($n = 8$), S6K1 ($n = 8$), and 4E-BP1 ($n = 5$). Each plotted shape and color represents the same participant or condition as indicated. Females ($n = 3$) are represented by filled black shapes and males ($n = 5$) by white shapes. Bars represent mean fold change ± SE of the mean error bars. All protein corrected to total (Ponceau staining) and log transformed (base 10). *Significant difference compared with baseline for indicated protein. *I*: representative images of protein expression for all protein targets at baseline (pre), 30 min post, and 3 h posttrial for RE, HS, and REH and total protein stain. Analyzed via linear mixed-effects ANOVAs. P indicates phosphorylated protein form. Analyzed via repeated-measures or mixed-effects ANOVAs. Akt, protein kinase B, mTOR, mechanistic target of rapamycin, S6K1, ribosomal S6 kinase beta 1, 4E-BP1, eukaryotic elongation initiation factor 4E-binding protein 1.

signaling factors related to heat shock and muscular hypertrophy in humans. In partial agreement with our hypotheses, our thermoregulatory results demonstrate that RE, HS, and REH similarly increased muscle but not core temperatures. Furthermore, REH increased absolute heat shock protein expression, while RE and HS did not. In addition, REH but not RE or HS increased absolute mTOR phosphorylation (Fig. 3). Alternatively, HS and REH but not RE increased peak heat shock protein expression in skeletal muscle, yet the effects of REH were not greater than HS alone (Fig. 3). Moreover, and in opposition to our hypothesis, HS resulted in greater peak activation of the Akt-mTOR cascade compared with RE and REH that did not provide any additional effects (Fig. 4). In partial agreement with our hypotheses, results from our cellular experiment demonstrate that acute heat stress enhanced myotube development to a lesser degree than growth media stimulated hypertrophy but to a greater degree than control and rapamycin treatment induced atrophy (Fig. 5). These early findings provide novel comparative data regarding thermoregulatory responses as well as skeletal muscle heat shock and hypertrophy-related signaling responses to acute whole body heat stress and resistance exercise, and their combination in humans. In addition, our cellular results provide supplemental evidence regarding the individual effects of heat stress in isolated skeletal muscle cells.

Thermoregulatory Responses

Body temperature responses to heat stress (4) and aerobic exercise (23) have been well studied compared with resistance exercise (24). Both passive whole body heat exposure and prolonged endurance exercise can raise core tempera-

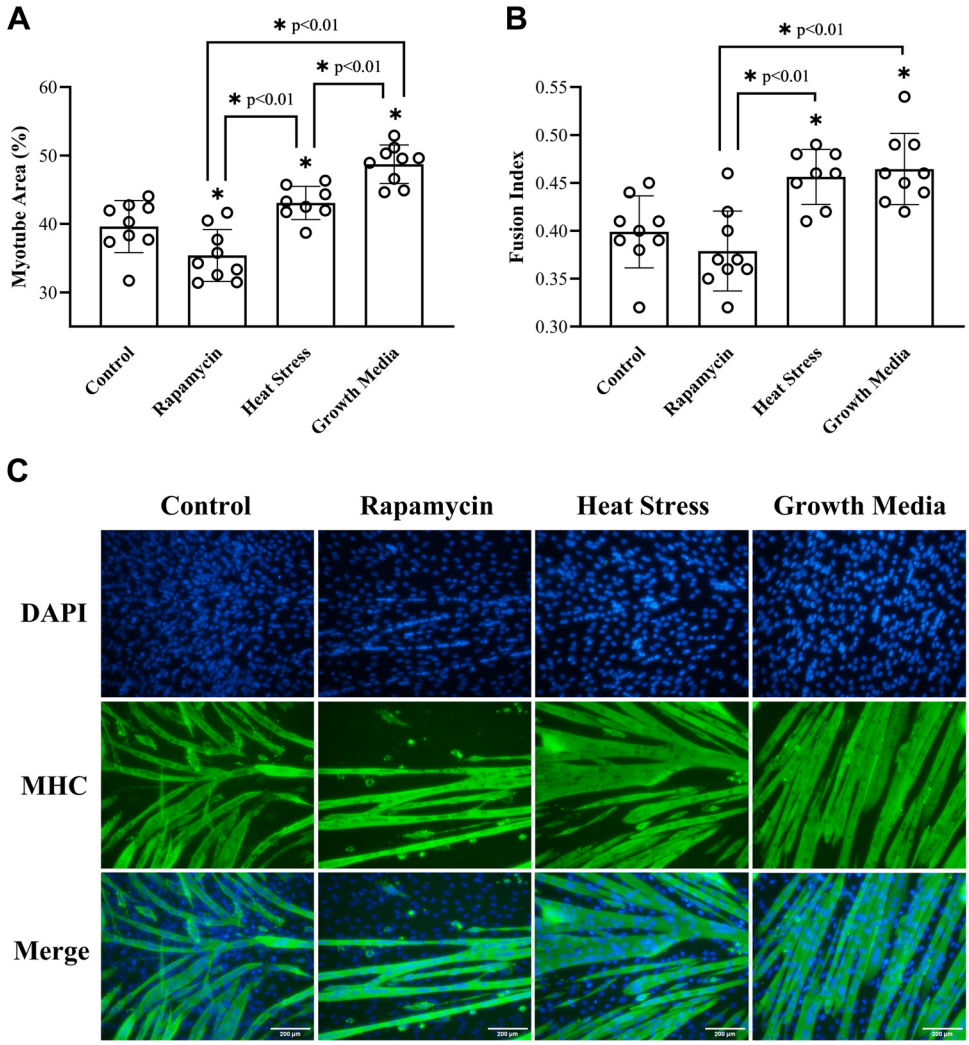

Figure 5. Cell culture outcomes. *A*: myotube area for control treated, rapamycin-treated, heat stress, and growth media-treated cells. *B*: fusion index for control, rapamycin-treated, heat stress, and growth media-treated cells. Individual points represent averaged values for individual replicates and white bars group averages with error bars as standard deviation. *C*: representative images for control, rapamycin, heat stress, and growth media-treated cells. DAPI: nuclear staining in blue, MHC: myosin heavy chain staining in green, Merge: merged DAPI and MHC staining. Scale bar represents distance of 200 μm. *Significant effect of condition compared with control cells, asterisk over bar significant difference between indicated conditions. Assessed via mixed-effects ANOVA with Holm–Sidak multiple comparison where relevant; *A*: rapamycin ($P = 0.02$), heat stress ($P = 0.03$), growth media ($P < 0.01$), rapamycin vs. heat stress ($P < 0.01$), rapamycin vs. growth media ($P < 0.01$), heat stress vs. growth media ($P < 0.01$); *B*: rapamycin ($P = 0.44$), heat stress ($P = 0.02$), growth media ($P < 0.01$), rapamycin vs. heat stress ($P < 0.01$), rapamycin vs. growth media ($P < 0.01$), heat stress vs. growth media ($P = 0.65$).

tures above 39°C (4, 23), depending on the magnitude of thermal stress and exercise environment, respectively. Conversely, evidence suggests some forms of resistance exercise have minor effects on core temperature, stimulating increases of less than half a degree (24). Our findings agree, showing that RE increased core temperature by ~0.5°C, which was significantly lower than the responses to HS (~1.3°C) and REH (~1.2°C) (Fig. 5). We expected the greatest responses from REH but there was no significant difference between HS alone or REH on thermoregulatory outcomes. Nonetheless, core temperature increases were roughly 0.6°C lower when comparing the heating portion of REH to HS. Yet, analysis of only the heating sessions indicated no significant core temperature difference between REH and HS conditions. We acknowledge that data artifacts could partially explain the observed difference in temperature gain but cautiously suggest the potential for a thermoregulatory priming effect of exercise performed before passive heat exposure. Accordingly, although elevations in core temperature before exercise are considered disadvantageous (25), the effects of resistance exercise including increased blood flow, sweat rate, and ultimately evaporative cooling, before passive heating could allow for greater evaporative cooling potential against an external heat load (25). Importantly, our results do not demonstrate this effect, and our current experimental design was not meant to pursue this line of inquiry, though it may merit further investigation.

Some findings suggest that acute resistance exercise results in minor (~1°C) changes to muscle temperature (24) after single muscle group exercises, whereas data from Roberts et al. (26) suggest increases as high as ~2–3°C following high-intensity resistance exercise. Our data demonstrate muscle temperature increases of ~2.0°C after RE, which was not statistically different from the effects of HS (~2.5°C) or REH (~3.2°C) despite greater average increases. If group differences were present, they may have been lost alongside high variability in participant responses as the range of muscle temperature increases was 0.2–5.6°C across all trials. In addition, a greater increase in muscular temperature may in part be due to the employed exercise protocols including higher volume and intensity whole body exercise (26) compared with low volume or isolated exercise sessions (24). Regardless, the fact that RE increased muscle temperature

similarly to HS raises questions regarding the potential role of muscular temperature for hypertrophic adaptations to exercise. The necessity of increased muscle temperature for growth has not been explicitly demonstrated; however, cold stress has been shown to impair hypertrophic adaptations in skeletal muscle (27, 28). In fact, cultured human myotubes exposed to 48 h of cold stress (32°C) demonstrate impaired morphologies, as well as nutrient-mediated mTOR pathway signaling, and protein synthesis (27). Moreover, repeated postresistance exercise cooling reduces anabolic signaling, strength gains, hypertrophy, and heat shock responses in humans (28). Despite this, the lack of significant heat shock and hypertrophy-related signaling responses for our RE group suggest that in a trained population, muscle temperature is not a key factor mediating acute hypertrophic signaling responses. Nonetheless, greater evidence is necessary to determine the role muscle temperature could play in hypertrophic signaling and adaptations, and whether connections between heat stress, HSP expression, and hypertrophy-related signaling exist.

Heat Shock Responses

When examining the absolute protein responses across the 30-min and 3-h time points, only REH increases HSPA expression at 30 min compared with baseline in resistance-trained but nonheat-acclimated humans (Fig. 3). Alternatively, when examining the peak responses occurring at either 30 min or 3 h, our results show that HSPA is increased following HS and REH but not RE. Furthermore, absolute HSPC responses are highly variable, demonstrating no significant effects for any condition across time points (Fig. 3). Conversely, peak HSPC expression is less variable and is decreased following HS but not RE or REH. It would stand to reason that whole body and tissue-specific temperature responses are important for the local induction and expression of various HSPs, particularly in skeletal muscle (11, 29), yet evidence in this regard is not unanimous (30). Previous data have revealed that whole body heating but not single-leg hot water immersion increases the skeletal muscle gene expression of HSPA and HSPC (4). Furthermore, whole body heating increased both core and quadriceps temperatures while single-limb heating only increased muscle temperature (31). As HS and REH in our experiment similarly resulted in greater core temperature responses than RE despite nonsignificant increases in muscle temperature, it is possible that whole body thermoregulatory strain is a stimulus for the local induction of HSPA. Accordingly, previous findings suggest that sustained elevations in core temperature are an important mediator of heat acclimation and HSPA expression in circulation (32). Yet, as only REH confidently increases HSPA across analyses, the time of heating application (e.g., across exercise and whole body heating) may additionally be relevant. Although the present study cannot adequately answer these questions, heat stress here and in other human, cell, and animal investigations increases HSP gene and protein expression to varying degrees (4, 7, 33–36), suggesting the magnitude of temperature responses are likely relevant. Conversely, some have shown increasing core (~1.5°C) and muscle (~3.6°C) temperatures do not influence the expression of HSPA in human skeletal muscle 48 h postheating (30). Yet, as we demonstrate peak increases for HSPA expression within 3 h of heat stress, acute signaling may be of greater relevance for this HSP. Conversely, others have demonstrated that 30 min of hot water immersion ranging from 37 to 41°C does not immediately increase the skeletal muscle expression of HSPA or HSPC in mice (5). Irrespective of these differences, the effects of HS alone observed here agree with evidence suggesting that heat can increase HSPA gene and protein expression in human skeletal muscle (4, 9). Of note, others have shown acute whole body heat stress can comparably increase the gene expression of HSPC in human skeletal muscle (4) while others suggest HSPC is unresponsive to heat stress in isolated cellular models (37). Though severely understudied in human skeletal muscle, HSPC expression may be less relevant for hypertrophic-related signaling events than HSPA. Nonetheless, HSP gene responses are not absolutely congruent to posttranscriptional exercise responses and in our sampling timeframe, HSPC protein expression is decreased in response to HS or the least unresponsive to our protocols.

In contrast to our findings, previous research demonstrates that resistance exercise increases HSPA protein expression in humans (38, 39). Importantly, the limited number of studies using damage-inducing training via downhill running (38) or chronic resistance training protocols in humans (39), make direct comparisons to our study complicated. Furthermore, the participants from these investigations were less trained compared with our population. As acute and chronic exercise of various types increases HSPA in human skeletal muscle (40), it is possible that our trained participants possessed higher basal HSP expression or blunted HSP responses following RE. This is corroborated by evidence in rats demonstrating a reduction in acute postexercise skeletal muscle HSPA expression following 8 wk of resistance training (41). Accordingly, as we demonstrate increases in HSPA expression following REH but weaker effects from HS, it is expected these responses would be further pronounced in untrained individuals. Regardless, our design allows for direct comparison between interventions and indicates acute resistance exercise does not acutely stimulate HSPA protein expression to the same degree as whole body heat stress plus resistance exercise and heat stress alone in a resistance-trained group. Of consideration, heat acclimation is well known for its ability to increase thermotolerance and the expression of HSPs (11, 42). As our participants were resistance trained but heat naive, the novel stimulus induced by heat stress alone or combined with resistance exercise could account for differences in HSPA response. The blunting and decay of HSP responses to and following heat acclimation are unknown and it is unclear if skeletal muscle heat shock responses would decrease with repeated exposure.

To our knowledge, no study has yet compared the effects of resistance exercise to heat stress on muscular heat shock protein expression in humans. Conversely, cell and animal models have demonstrated similar responses when comparing exercise or exercise-like stimuli and heat stress (7). In fact, in cultured rat myotubes undergoing cyclic stretching (96 h) or heat stress (60 min, 41°C), HSPA and HSPC expressions were increased to a similar degree (7). Another investigation induced soleus overload in rats and showed overload alone increased HSPA expression in skeletal muscle within 3 days, while acute heat stress (15 min, core temperature

maintained at 42°C) resulted in a comparatively greater HSPA protein expression within 1 day (35). While comparisons between cell, animal, and human models are quantitatively imperfect, these data are contrary to ours, indicating both contraction and heat stress are capable of eliciting HSP responses. Although transcriptional and translational HSP responses may be discordant, more research is required to determine how resistance exercise influences HSP activity in human skeletal muscle and how training status, exercise intensity or volume, and measurement time frame might influence the outcomes.

Hypertrophy-Related Signaling

Our results show that in a resistance-trained, but heat-naive population, acute HS and REH increased the phosphorylation status of mTOR when examining peak responses or across 30 min to 3-h time points in skeletal muscle (Fig. 4). Alternatively, analysis of peak responses suggests HS can increase S6K1 phosphorylation. Through activation of growth pathways including the Akt-mTOR cascade, resistance exercise and growth-stimulating conditions increase myofibrillar protein synthesis, resulting in greater muscle cross-sectional area (1). Although not absolutely necessary for maintenance, multiple investigations implicate that this signaling pathway maintains muscular function and stimulates hypertrophic responses (43). Therefore, despite an inability to demonstrate increased myofibrillar protein synthesis or muscular growth, our results suggest that prohypertrophic signaling responses were greater following HS and REH compared with RE as indicated by the increased phosphorylation status of the Akt-mTOR cascade. Furthermore, HS alone may further enhance the phosphorylation of S6K1, suggesting additional downstream activation compared with REH. Cautiously, these findings suggest heat stress alone or in combination with resistance exercise enhances hypertrophic signaling responses through the Akt-mTOR cascade in a resistance-trained population.

Resistance exercise is well established as a hypertrophy-inducing stimulus, and exercise protocols similar to ours increase myofibrillar protein synthesis and skeletal muscle mass over time (44). In this regard, our RE protocol served as a comparative control known to induce muscle hypertrophy with progressive application. Though we hypothesized a greater effect of RE alone on Akt-mTOR-mediated signaling, we only observed significant activation following HS and REH. Although unexpected, individuals with prior strength training history, like those in our study, have demonstrated blunted hypertrophy-related signaling responses to resistance exercise (44, 45). In fact, it has been suggested that acute responses including myofibrillar protein synthesis rate do not predict skeletal muscle hypertrophy in trained individuals (45). In agreement, the acute phosphorylation status of Akt, mTOR-inhibiting tuberous sclerosis complex 2, and S6K1 are unchanged in trained individuals contrary to their activation in untrained but physically active individuals (46). We also acknowledge that muscle hypertrophy is not solely dependent on Akt-mTOR-associated signaling. Growing perspectives propose divergent hypertrophic pathways including myostatin and transforming growth factor-activin receptor-related pathways (47). Furthermore, 4E-BP1 has been demonstrated unessential for muscle hypertrophy in response to mechanical overload in mice (48). Nevertheless, inhibition or knockout of the primary Akt-mTOR factors is detrimental to muscular development and growth (43). Moreover, mTOR explicitly is a key mediator of muscular growth, which can be activated through Akt-independent mechanisms resulting in the gene expression of eukaryotic initiation factor 2B (49). This is particularly notable as muscle protein synthesis rates following resistance exercise in rats display greater dependence on eukaryotic initiation factor 2B activity than 4E-BP1 (49). Thus, lacking increased activation of Akt or 4E-BP1 following RE or HS, we show that only protocols including heat stress stimulated phosphorylation of the upstream control point, mTOR, and possibly S6K1, in a trained population. Others have previously presented hypertrophic (1, 6, 7, 33) and related signaling (4, 5, 15, 33) effects following various forms of heat stress, with some mixed results (31, 35, 50, 51). Our findings agree with those suggesting a hypertrophic signaling effect following heat stress. For example, a comparable heat stress model utilizing 60 continuous min of whole body heat stress (45–50°C, 50% relative humidity) found increased phosphorylation status compared with baseline for similar acute markers including Akt, mTOR, S6K1, and eukaryotic initiation factor 4E (4). This is matched by various cell and animal investigations cumulatively indicating heat stress increases the activity of the Akt-mTOR cascade (5, 36). Despite the increased signaling response of REH and possibly HS compared with RE, we have not explored the chronic effects of heating, and to date, few studies have demonstrated a gross hypertrophic effect of heat stress alone in humans. Accordingly, it should be considered that heat stress may assist in the activation of Akt-mTOR signaling but not explicit myofibrillar protein synthesis. In this case, heat stress could be a stimulus for global protein synthesis but not the distinct proteins leading toward gross muscular hypertrophy. Yet, as muscle cell-specific protein aggregation or growth has been previously demonstrated in cellular and animal models (7, 33, 34, 36), this distinction requires further exploration in humans.

Importantly, differences in experimental design including heating methodology and timing should be noted. A number of human experiments have examined the effects of heat stress plus resistance exercise on muscular hypertrophy (6, 51) or related signaling (50) with varied effectiveness compared with resistance exercise alone (50, 51). These studies have included a range of training intensities and loads, which could explain some discrepancies in findings. For example, direct heating enhances triceps hypertrophy following low-load training (3 × 30, <30% 1 RM) plus direct heating (6) but adds no benefit to quadriceps growth compared with high-load (4 × 8, 70% 1 RM) resistance training alone (51). Our data agree to some extent, showing that the effects of high-load resistance training on Akt-mTOR activation were surpassed by heat stress but not further enhanced by the addition of resistance exercise and heat stress. Furthermore, mTOR activation and myofibrillar protein synthesis were not different in experiments comparing high-load resistance exercise (4 × 10, 80% 1 RM) with or without the addition of heat stress (50). Importantly, this may support the idea that heat stress does not stimulate myofibrillar protein synthesis in the same manner as resistance exercise in humans. Notwithstanding, more research is necessary to determine if these effects result in meaningful outcomes and

if heat stress alone can progressively elicit gross muscle hypertrophy in humans. Furthermore, as discussed previously, the blunted Akt-mTOR responses following RE compared with REH and possibly HS may be partially accounted for by the participants' resistance training familiarity but heat naivety. This is particularly true when pairing the elevated HSP responses following REH to the potential for heat shock and hypertrophy-related signaling connections.

Heat Shock Connections

Previous research has indicated points of interaction between various HSPs, including HSPA and HSPC, and Akt-mTOR-mediated hypertrophic signaling (15, 16, 52, 53). HSPA appears to interact with mTOR complex 2 and is mechanistically relevant for subsequent Akt activation upon heat stimulation (52). In addition, HSPC can interact with mTOR complex 1 preventing phosphatase-mediated apoptosis (16), while HSP inhibition impairs the phosphorylation of S6K1 and 4E-BP1 (53). Moreover, evidence demonstrates that HSP reduction in mice, via upstream heat shock transcription factor 1 knockout, impairs the hypertrophic effects of heat stress (15). Focusing on an opposing set of mechanisms, HSPs inhibit atrophy-inducing mechanisms including the ubiquitin-proteasome and autophagy-lysosome systems and can decrease muscle atrophy (9, 10, 33, 54). Though unmeasured in the current investigation, HSP-mediated atrophy inhibition could have played a role and warrants future investigation. Regardless, our results only show that increases in skeletal muscle hypertrophy-related signaling occurred despite decreased or unchanged HSPC expression following HS and REH, respectively. Conversely, REH and to a less definitive state HS, increased the expression of HSPA while promoting greater hypertrophic signaling than RE. Importantly, we have acknowledged this response may be in part because the participants were resistance familiar but heat naive. These findings are undoubtedly interesting, yet we have not measured direct interactions between HSPs and Akt-mTOR proteins. Subsequently, our data can only indicate that changes in HSPA and potentially HSPC expression as well as activation of the Akt-mTOR cascade occurred in tandem following REH but not RE. Furthermore, though inconclusive, HS alone may be able to stimulate similar responses to REH. Greater evidence, including direct analyses, is needed to examine these effects and determine their mechanistic connections.

Cell Culture Outcomes

As in vivo measurements of muscle protein synthesis were not used and a chronic-training design was not practical for this investigation, we employed a simple cell culture model to examine the effects of heat stress on isolated myotubes. This included comparison of positive and negative cellular hypertrophy models induced by growth medium supplementation (10% FBS) and rapamycin (164 nM) treatment, respectively. Though imperfect, this served as a proxy model for the rapid resolution of cellular hypertrophy following heat stress compared with a growth stimulus and specific mTOR inhibitory atrophy model. Similar to previous reports (19), our results demonstrate that growth media treatment enhances myotube area and the number of nuclei contained within differentiated myotubes (fusion index) (Fig. 4). Likewise, we observed decreased myotube differentiation without a reduction in fusion index compared with control conditions following treatment with rapamycin (20). Furthermore, we show that a single bout of heat stress (40°C) increases myotube growth and fusion compared with untreated cells, albeit with lower myotube area but similar fusion index increases as growth media treatment. Moreover, as the number of total nuclei did not change across any condition, it suggests an increased myonuclear domain for growth media treatment and heat stress. Multiple animal and cell models have demonstrated hypertrophic effects of heat stress under various conditions (7, 33, 34, 36). Yet, few have included isolated hypertrophy or atrophy control conditions (7, 35). From these limited experiments, it appears that mechanical stimulation in cells enhances protein accumulation to a similar degree as heat stress (7) but does not increase rat muscle mass without limb overload (35). Our results partially agree with both findings, suggesting acute heat stress can enhance cellular hypertrophy within 48 h, albeit to a lesser degree than growth medium conditions. Similarly, others have shown that heat stress increases myonuclear development in cells and animals (33, 36, 55). In fact, 60 min of heat (42°C) increases the percentage of formed multinuclear myotubes by 7 days compared with controls (55). Interestingly, HSPA overexpression has been indicated as a promotor of C2C12 fusion and myotube diameter but not myoblast proliferation (37). Notably, as we have not measured the expression of HSPs in our C2C12 experiment, we can only speculate considering a heat shock-mediated mechanism presently.

Similar to our rapamycin-treated cells, cellular atrophy models have demonstrated a loss in myotube area without changes in total nuclei, indicating a reduction in myonuclear domain (56). While classically a focus for muscle development, atrophy, or regrowth models, evidence indicates a role of myonuclear development in postnatal muscle hypertrophy (57). The exact nature of myonuclear domain expansion to hypertrophy is still a topic of debate, yet growing evidence suggests nuclear development occurs alongside and is possibly necessary for muscular growth (57). Research has demonstrated individuals with greater hypertrophic responses to prolonged training presented profound satellite cell and myonuclear responses compared with lower responders (58). In line with these responses, a single 60-min heat session (42°C) is capable of increasing satellite cells alongside skeletal muscle mass in rats (33). Importantly, we have not measured satellite cell responses, yet our results demonstrate that heat stress stimulates changes in myotube fusion index comparable to growth media treatment and in contrast to the reductions induced by rapamycin treatment. While speculative, these findings indicate the potential for both myotube growth and myonuclear expansion, which may be relevant for elucidating the hypertrophic effects of heat stress in vitro and provide proof of concept for heat stress-induced muscle growth. Regardless, the in vivo results from this investigation should be viewed as indirect evidence alongside the human outcomes and cannot be directly associated with the current investigation.

Limitations

A primary limitation of the present study is the difference in duration across experimental trials. As we chose to match

the biopsies based on the trial endpoints, RE and HS sessions were approximately the same duration while the REH was effectively doubled. Accordingly, the efficacy of their comparisons, particularly considering protein signaling responses, should be considered. Despite this limitation, our comparisons of RE and HS are relatively matched, whereas the comparison of RE and HS to REH should be taken with greater caution. An additional limitation includes the fasted state of our participants. Even so, trained individuals present increased myofibrillar protein synthesis rates in response to fasted resistance exercise and activation of mTOR-related signaling cascades (59, 60). Regardless, the magnitude of effect considering Akt-mTOR responses might be influenced by the fasted state used here and limits applicability to fed-state conditions. In addition, the lack of a powerful sex comparison among our participants is a limitation (Supplemental Table S1). Others have demonstrated slight differences in Akt-mTOR-related signaling between males and females following resistance exercise (61) yet current literature indicates congruent signaling, protein synthesis, and adaptive responses irrespective of sex and developmental differences (62). Therefore, these differences did not appear to change major statistical outcomes in our study and suggest similar responses for males and females. In addition, while sex-specific responses such as menstrual cycle can increase resting core temperature, factors including contraceptive use and hormonal differences appear to have little influence on overall athletic performance (63). Accordingly, if seeking to appropriately characterize sex-specific responses or differences in skeletal muscle signaling events or thermoregulatory responses, future investigators should include larger cohorts of males and females to power their investigations in a manner specific to their line of questioning.

A primary limitation of our cell culture designs includes the lack of investigation of heat shock and hypertrophy-related protein expression. Nevertheless, others have shown that similar acute heat stress increases the expression of HSPA alongside changes in protein content or myotube development in vitro (7, 55). Future investigation including these analyses may help elucidate mechanisms of action considering the heat shock and hypertrophy connection. Finally, the results from our cellular investigation cannot be directly applied to our findings in humans. Though related in design and tissue domain, our cell model used immortalized mouse muscle cells and their independent results should be viewed with caution until empirically demonstrated across models. Furthermore, the newly formed mouse myotubes were unfamiliar with either heat stress or growth-stimulating conditions compared with our resistance-trained but heat-unfamiliar human participants who undoubtedly experienced regular hypertrophic signaling events and subsequent adaptation. Accordingly, the use of primary human cell lines, including multiple populations, would benefit current understandings in this field.

Perspectives and Significance

The results from this experiment demonstrate that acute whole body heat stress, resistance exercise, and resistance exercise followed by heat stress similarly increase core but not skeletal muscle temperature. Next, acute whole body heat stress plus resistance exercise increased absolute and peak skeletal muscle HSPA expression. Alternatively, HS alone increased the expression of HSPA and decreased HSPC expression using peak values only. Heat stress plus resistance exercise resulted in greater phosphorylation of the Akt-mTOR cascade than resistance exercise in humans across analysis techniques while HS only significantly increased activity when considering peak responses. Finally, our cellular experiments demonstrate that acute heat stress increased C2C12 myotube development, albeit to a lesser degree than growth media-stimulated conditions, while both heat stress and growth media conditions increased myonuclear fusion. This proof-of-concept model demonstrates that heat stress enhances muscular hypertrophy and development in isolated muscle cells. Together these findings suggest enhanced hypertrophic signaling when acute heat stress is used following resistance exercise in humans and limited evidence that heat stress alone can do the same. Conversely, heat stress alone has the potential to directly stimulate hypertrophy in muscle cells. Nonetheless, greater investigation is needed to determine if the acute effects of heat stress alone or in combination with resistance exercise can influence growth stimuli including myofibrillar protein synthesis and induce gross muscular hypertrophy in humans. Furthermore, it remains unclear if factors including training status and heat familiarity can modulate the hypertrophic effects of resistance exercise, heat stress, or their combination.

DATA AVAILABILITY

Data will be made available upon reasonable request.

SUPPLEMENTAL DATA

Supplemental Tables S1 and S2: https://doi.org/10.6084/m9.figshare.21804012.

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Figure 1 and graphical abstract were created with BioRender and published with permission.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Z.J.F., J.B.D., Q.N.B., Z.J.M., P.S.H., M.R.D., F.T.A., and C.M.M. conceived and designed research; Z.J.F., J.B.D., Q.N.B., J.W.S., Z.J.M., and S.E.S. performed experiments; Z.J.F. analyzed data; Z.J.F., J.B.D., Q.N.B., J.W.S., Z.J.M., R.C.N., K.A.E., P.S.H., M.R.D., F.T.A., and C.M.M. interpreted results of experiments; Z.J.F. and J.B.D. prepared figures; Z.J.F. drafted manuscript; Z.J.F., J.B.D., Q.N.B., J.W.S., Z.J.M., S.E.S., R.C.N., K.A.E., P.S.H., M.R.D., F.T.A., and C.M.M. edited and revised manuscript; Z.J.F., J.B.D., Q.N.B.,

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