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RESEARCH ARTICLE | *Physiology of Thermal Therapy*

Effects of repeated local heat therapy on skeletal muscle structure and function in humans

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Kim K, Reid BA, Casey CA, Bender BE, Ro B, Song Q, Trewin AJ, Petersen AC, Kuang S, Gavin TP, Roseguini BT. Effects of repeated local heat therapy on skeletal muscle structure and function in humans. *J Appl Physiol* 128: 483–492, 2020. First published January 23, 2020; doi:10.1152/japplphysiol.00701.2019.—The purpose of the present study was to examine the effects of repeated exposure to local heat therapy (HT) on skeletal muscle function, myofiber morphology, capillarization, and mitochondrial content in humans. Twelve young adults (23.6 ± 4.8 yr, body mass index 24.9 ± 3.0 kg/m^2) had one randomly selected thigh treated with HT (garment perfused with water at ~52°C) for 8 consecutive weeks (90 min, 5 days/wk) while the opposite thigh served as a control. Biopsies were obtained from the vastus lateralis muscle before and after 4 and 8 wk of treatment. Knee extensor strength and fatigue resistance were also assessed using isokinetic dynamometry. The changes in peak isokinetic torque were higher ($P = 0.007$) in the thigh exposed to HT than in the control thigh at *weeks 4* (control 4.2 ± 13.1 Nm vs. HT 9.1 ± 16.1 Nm) and *8* (control 1.8 ± 9.7 Nm vs. HT 7.8 ± 10.2 Nm). Exposure to HT averted a temporal decline in capillarization around type II fibers ($P < 0.05$), but had no effect on capillarization indexes in type I fibers. The content of endothelial nitric oxide synthase was ~18% and 35% higher in the thigh exposed to HT at 4 and 8 wk, respectively ($P = 0.003$). Similarly, HT increased the content of small heat shock proteins HSPB5 ($P = 0.007$) and HSPB1 ($P = 0.009$). There were no differences between thighs for the changes in fiber cross-sectional area and mitochondrial content. These results indicate that exposure to local HT for 8 wk promotes a proangiogenic environment and enhances muscle strength but does not affect mitochondrial content in humans.

NEW & NOTEWORTHY We demonstrate that repeated application of heat therapy to the thigh with a garment perfused with warm water enhances the strength of knee extensors and influences muscle capillarization in parallel with increases in the content of endothelial nitric oxide synthase and small heat shock proteins. This practical method of passive heat stress may be a feasible tool to treat conditions associated with capillary rarefaction and muscle weakness.

heat therapy; skeletal muscle

INTRODUCTION

Repeated exposure to whole body passive heat therapy (HT) in the form of hot water immersion, sauna, or environmental chambers has been shown to promote a plethora of health benefits in young individuals (4, 5, 7, 19) as well as in elderly patients with chronic heart failure (30, 35) and other cardiovascular diseases (20, 34, 36). For example, a recent population-based study revealed that frequent sauna bathing is associated with a significantly lower risk of fatal cardiovascular disease events and all-cause mortality (26). The salutary effects of HT are thought to stem in part from beneficial changes in the cardiovascular system, including improved endothelial function and reduced arterial stiffness and blood pressure (4, 5). However, it is increasingly evident that HT also elicits positive changes in skeletal muscle structure and function. Treatment with whole body HT for 6 wk increased skeletal muscle capillary density and endothelial cell-specific endothelial nitric oxide synthase (eNOS) content in young individuals (19). Moreover, as few as 11 days of daily exposure to heat stress in an environmental chamber improves skeletal muscle contractility, as evidenced by an increase in evoked peak twitch amplitude and maximal voluntary torque production (32).

Although whole body HT modalities have received the greatest attention, emerging evidence indicates that local HT may also promote skeletal muscle remodeling in humans. Both superficial (e.g., hot packs, heat wraps, water-circulating garments) and deep tissue (e.g., short-wave diathermy) local HT modalities are extensively used in rehabilitation settings for the management of muscle injuries as well as other conditions associated with pain and stiffness (28, 29). In contrast to whole body HT, heating of a small area or body segment typically induces minimal or no change in body core temperature. Goto and coworkers first reported that repeated local thigh heating increased isometric force production of the knee extensors in humans (13). Hafen and coworkers reported that short-term heat treatment promotes mitochondrial adaptations (16) and attenuates immobilization-induced atrophy in human skeletal muscle (15). We previously demonstrated that a single session of local thigh heating enhances the mRNA expression of factors associated with vascular growth, including vascular endothelial growth factor (VEGF) (25). Together, these studies indicate that local HT may be a practical tool to enhance skeletal muscle mitochondrial content and capillarization and improve contractile function. Nonetheless, the long-term skeletal muscle adaptations to repeated local heat stress in humans remain poorly defined.

The goal of the present study was to comprehensively examine the effects of 8 wk of exposure to local HT (5

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days/wk) on muscle strength, myofiber morphology, capillarization, and mitochondrial content in humans. Healthy young adults had one randomly selected thigh treated with HT using a water-circulating garment perfused with water at ~52°C for 90 min, while the opposite thigh served as a control. This heat modality and protocol were selected because *1*) a single 90-min session of local HT increases the skeletal muscle expression of heat shock proteins and angiogenic factors (25) and *2*) five daily 90-min sessions of local HT hastens functional recovery after eccentric exercise-induced muscle damage (22). On the basis of these previous findings, we hypothesized that daily exposure to heat stress would enhance muscle strength, promote muscle capillary growth and the expression of angiogenic mediators, and increase muscle mitochondrial content.

METHODS

Participants. Twelve healthy young adults (10 men, 2 women) volunteered to participate in this study (mean ± SD: 23.6 ± 4.8 yr, 172.9 ± 8.6 cm, 74.5 ± 10.3 kg). Participants were asked to fill out a health and medical history questionnaire before enrollment. Exclusion criteria were pregnancy, obesity (body mass index > 30 kg/m^2), hypertension (resting systolic/diastolic blood pressure > 140/90 mmHg), smoking, intake of medications and vitamin supplements, and history of deep vein thrombosis. Individuals who participated in any kind of supervised physical activity or engaged in physical activity >3 days a week were also excluded. Participants were informed about risks and discomforts related to the different tests and procedures of the study before providing their written informed consent to participate. The experimental procedures adhered to the standards in the latest revision of the Declaration of Helsinki and were approved by the Institutional Review Board at Purdue University (1604017606).

Experimental design. Participants initially visited the laboratory on four separate occasions over a 2- to 3-wk period. On *visits 1* and *2*, participants were familiarized with muscle testing on the isokinetic dynamometer. On *visit 3*, participants underwent the baseline assessment of muscle strength and fatigability as described in detail below. These initial testing sessions were separated by a minimum of 48 h. At least 1 wk after *visit 3*, resting muscle biopsies were collected from the vastus lateralis of the left and right legs of each subject (22, 25). The 8-wk intervention protocol commenced at least 3 days after the muscle biopsy procedures. In a within-subject design, the legs of participants were assigned in a counterbalanced fashion to receive HT or no treatment. Participants were asked to report to the laboratory 5 days/wk for a total of 40 sessions. The length of the intervention (8 wk) was based on the reports by Brunt and coworkers that 8 wk of whole body HT improves conduit vessel and cutaneous microvascular function (4, 5). Muscle strength and fatigability were reassessed after 4 and 8 wk of treatment. These experimental sessions took place ~24 h after the previous HT session. At least 48 h after the completion of muscle testing, muscle biopsies were taken from each thigh.

All visits were conducted in an environmentally controlled laboratory at a similar time of day. Participants were instructed to fast for 10–11 h before undergoing muscle biopsies and to eat a light meal before the other experimental visits. Participants were instructed to abstain from vigorous physical activity in the 24 h preceding each test and to avoid caffeine consumption on the day of testing. Participants were asked to maintain their normal dietary and exercise behavior throughout the study. At the end of each week, participants were asked to self-report the frequency, duration, and intensity of physical activity performed in the preceding 5 days.

Heat treatment. Participants were asked to report at the same time of day for the treatment sessions. Upon arrival at the laboratory, thermocouples (MLT422; ADInstruments, Colorado Springs, CO) were taped to both thighs for measurement of skin temperature. Participants were asked to put on water-circulating trousers on top of shorts or underwear (Med-Eng, Ottawa, ON, Canada). This garment was customized with an extensive network of medical-grade polyvinyl chloride tubing that covered the thighs and buttocks (22, 25). In the thigh assigned to receive HT, water at ~52°C was perfused through the garment for 90 min with a goal of increasing leg skin temperature to ~39.5–40°C (22, 25). Previous studies that employed a similar approach revealed that this regimen causes muscle temperature to increase from a baseline of ~33–34°C to ~37°C (8, 17).

Assessment of muscle strength and fatigability. Knee extensor strength and fatigue resistance were assessed with an isokinetic dynamometer (Humac NORM; Computer Sports Medicine, Stoughton, MA) as described previously (22). Participants were familiarized with the testing procedures twice before the baseline assessment. Participants were seated with hands across the chest, restraining straps over the trunk, pelvis, and thigh, and the input axis of the dynamometer aligned with the axis of rotation of the knee. The familiarization protocol included a set of 5–10 concentric knee extension contractions at 60–70% of the estimated maximal effort at an angular velocity of 180°/s, a set of three maximal contractions at 180°/s, and a set of 40 consecutive maximal contractions at 180°/s.

In each experimental session, participants were allowed to warm up for 5 min on a cycle ergometer and were then positioned on the chair of the isokinetic dynamometer with the identical apparatus setting predetermined at the first familiarization visit. Testing was performed on both legs, with the order of the testing counterbalanced among participants. Participants were asked to complete three maximal consecutive contractions at 180°/s, with a resting period of 3 min between limbs. The maximal measured torque (Nm) was used in all analyses. Once both limbs had been tested for maximal strength, participants were allowed to rest for ~3 min and were then asked to perform a bout consisting of 40 consecutive maximal contractions at 180°/s. A resting period of 10 min was allowed between limbs. The total work (J) performed during the bout was computed and used as a measure of fatigue resistance of the knee extensors. The investigator who conducted the assessment of muscle function was not blinded to the treatment assignment.

Muscle sampling. Muscle biopsies were obtained from the vastus lateralis under local anesthesia (lidocaine hydrochloride; Hospira, Lake Forest, IL) with a 5-mm Bergström biopsy needle (Pelomi Medical, Albruslund, Denmark). The biopsy specimens were promptly weighed, cleared of visible fat and connective tissue, and divided into three sections. Approximately 40-mg sections were mounted in transverse orientation in a disposable base mold with an embedding medium compound (Tissue-Tek O.C.T. compound; Sakura Finetek USA, Torrance, CA) and then frozen in liquid nitrogen-cooled isopentane for cryosectioning. The other sections were immediately frozen in liquid nitrogen and stored at −80°C until citrate synthase and Western blot analysis.

Immunohistochemistry. Transverse serial sections (10 μm) of muscle were cut with a Leica CM1850 cryostat (Leica, Wetzlar, Germany) at −23°C, mounted on frosted microscope slides (Thermo Scientific, NH), air-dried for 0.5–1 h at room temperature, and stored at −80°C for subsequent analyses. Frozen sections were briefly exposed to room air and fixed with 4% paraformaldehyde for 5 min. After 2 × 3 min washes with 1× PBS, the slides were incubated with blocking buffer (5% goat serum, 2% bovine serum albumin, 0.1% Triton X-100, and 0.1% sodium azide in PBS) for 1 h at room temperature.

Muscle fiber type distribution was probed with primary antibodies against the basal lamina and myosin heavy chain (MHC) isoform proteins. Sections were incubated for 3 h at room temperature with the following primary antibodies: polyclonal rabbit anti-laminin IgG (ab11575, 1:500; Abcam), monoclonal mouse anti-MHC I IgG2b (BA-D5, 1:100), monoclonal mouse anti-MHC IIa IgG1 (A4.74, 1:100), and monoclonal mouse anti-MHC IIx IgM (6H1, 1:100). All MHC primary antibodies were purchased from the Developmental

Studies Hybridoma Bank (University of Iowa, IA). After incubation, tissue sections underwent a series of 1× PBS washes and incubation with fluorescently labeled secondary antibodies for 1 h at room temperature: Alexa Fluor 488 goat anti-rabbit IgG (A11008, 1:1,000), Alexa Fluor 488 goat anti-mouse IgG2b (A21141, 1:1,000), Alexa Fluor 568 goat anti-mouse IgG1 (A21124, 1:1,000), and Alexa Fluor 350 goat anti-mouse IgM (A31552, 1:1,000). All secondary antibodies were obtained from Thermo Fisher Scientific. After 4 × 5 min washes, slides were briefly dried and mounted with fluorescent mounting medium (Dako, CA) and the edges were sealed with nail polish (Sally Hansen Hard as Nails, NY).

Identification of fiber type-specific capillaries was performed in neighboring sections with antibodies against mouse anti-CD31 IgG1 (550300, 1:100; BD Biosciences), rabbit anti-dystrophin IgG1 (ab15277, 1:100; Abcam), and mouse anti-MHC I (BA-D5, 1:100; Developmental Studies Hybridoma Bank). After 2 × 5 min washes with 1× PBS, sections were stained with appropriate secondary antibodies [Alexa 350 goat anti-rabbit IgG (A11609, 1:500), Alexa 488 goat anti-rabbit IgG (A11008, 1:1,000), Alexa 488 goat anti-mouse IgG 2b (A21141, 1:1,000), and Alexa 568 goat anti-mouse IgG1 (A21124, 1:10,000); Thermo Fisher Scientific] diluted in 1× PBS for 1 h at room temperature. Negative controls for the primary antibodies against CD31 were used to ensure specificity of staining.

Slides were viewed at ×20 magnification with an Olympus BX53 fluorescence microscope equipped with an Olympus DP72 digital camera and cellSens Dimension software. The entire specimen cross section was initially selected with the stage navigator. The multichannel image was then acquired, and two images from each channel were merged with ImageJ software (National Institutes of Health). Histological analysis was not performed in 1 of 72 samples because of insufficient muscle yield.

Analysis of immunofluorescence images. Analyses of immunofluorescence images were carried out with Adobe Photoshop CC 2015. Fiber type distributions were determined from counts of an average of 612 ± 70 muscle fibers (range 221–1,260 fibers). For the quantification of muscle capillarization, all internal fibers (not bordering on a fascicle) in a cross section were initially counted (an average of 130 ± 23 fibers for type I and 153 ± 26 fibers for type II muscle fibers). A total of 25 type I and 25 type II muscle fibers were then randomly selected for analysis. Individual fibers were traced to obtain the area and perimeter of the fiber. Capillaries were quantified with the following indexes: *1*) the number of capillaries around a fiber (capillary contacts, CC), *2*) the capillary-to-fiber ratio on an individual fiber basis (C:Fi) and *3*) the number of fibers sharing each capillary (sharing factor, SF), and *4*) the capillary-to-fiber perimeter exchange index (CFPE index), defined as C:Fi divided by the fiber perimeter of a given fiber (18). All immunofluorescent images were blinded for both treatment and time point before analysis.

Protein extraction. Frozen muscle samples (~30 mg) were homogenized in ice-cold homogenization buffer containing 50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, and 1 mM EDTA (RIPA Lysis Buffer; EMD Millipore), with freshly added protease inhibitor cocktail (P8340; Sigma-Aldrich) and phosphatase inhibitors (50 mM NaF and 0.2 mM Na_3VO_4) at a 1:15 dilution of wet muscle weight with a bead mill homogenizer (Bead Ruptor 12; Omni International). The resulting homogenate was clarified by centrifugation (13,500 *g*) for 20 min at 4°C. The supernatant was collected, and the protein concentration of each sample (~5 μg/μL) was determined with a BCA protein assay kit (Thermo Scientific, IL). All samples were subsequently diluted with homogenization buffer (1.5 μg/μL) and subsequently mixed with either reducing sample buffer (4× Laemmli sample buffer with 10% 2-mercaptoethanol) or nonreducing sample buffer (4× Laemmli sample buffer). Afterwards, samples were heated to 95°C for 5 min (except for mitochondrial oxidative phosphorylation (OXPHOS) protein blots), divided into small aliquots, and stored at −80°C.

Western blot analysis. For the analysis of HSP90A, HSP90B, VEGF, ANGPT1, eNOS phosphorylated at Ser1177 (p-eNOS^{ser1177}), eNOS, and OXPHOS, 20 μg of protein was separated by SDS-PAGE on precast Stain Free 4–15% gels (Bio-Rad, CA) and transferred to polyvinylidene fluoride membranes with the Trans-Blot Turbo transfer system (Bio-Rad, CA). Membranes were subsequently blocked with 5% nonfat milk in 1× Tris-buffered saline-Tween (TBST, 1% Tween 20) for 1 h at room temperature (~23°C) and incubated for 3–4 h at room temperature with primary antibodies diluted in blocking buffer. The membranes were washed with 1× TBST at room temperature for 3 × 10 min, incubated with horseradish peroxidase-conjugated secondary antibodies diluted in 1× TBST for 1 h at room temperature, and then washed with 1× TBST at least 3 × 10 min before being exposed to an enhanced chemiluminescent solution (Clarity Western ECL; Bio-Rad) for 5 min. Membranes were visualized with a densitometer (ChemiDoc Touch Imaging System; Bio-Rad), and band densities were determined with image analysis software (Image Laboratory v.6.0.1; Bio-Rad). PageRuler Prestained Protein Ladder (Thermo Fisher) was used as a molecular weight marker. Control for equal loading was performed with stain-free technology, and total protein normalization was used to calculate changes in the expression of each target protein relative to the baseline sample. The analysis of HSPB5, HSPB1, and HSPA1A was performed as described previously (11). Details of the primary antibodies are provided in Supplemental Table S1 (see https://doi.org/10.6084/m9.figshare.11385921). Recombinant proteins were used to confirm antibody specificity.

Citrate synthase activity. The maximal enzyme activity of citrate synthase (CS) was determined with the lysate prepared for Western blot analyses and analyzed on a spectrophotometer (Bio-Rad). Samples were analyzed in triplicate, and each well (final reaction volume 210 μL, path length 0.57 cm) contained 10 μL of ~2 mg/ml lysate, 0.3 mM acetyl-CoA, 0.15 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 0.25% (wt/vol) Triton X, and 1 mM oxaloacetate made to volume with 100 mM Tris buffer, pH 8.3. Oxaloacetate was added to commence the reaction, which was measured by change in absorbance (DTNB ε = 14,150 $M^{-1}\cdot cm^{-1}$ at 412 nm) every 15 s over a 3 min period at 25°C; enzyme activity was then expressed as nanomoles per minute per milligram of protein.

Statistical analysis. All statistical analyses were conducted with SAS (version 9.4; SAS Institute, Cary, NC), with results expressed as means ± SD. The Kolmogorov–Smirnov test was used to assess the distribution of the data. Data exhibiting skewed distribution (HSPB5, HSPB1, p-eNOS, ANGPT1, HSP90A, HSP90B) were log-transformed before statistical analysis. Descriptive results for each variable are expressed as means ± SD or geometric mean × / ÷ geometric standard error if the variable value was log-transformed. A two-way

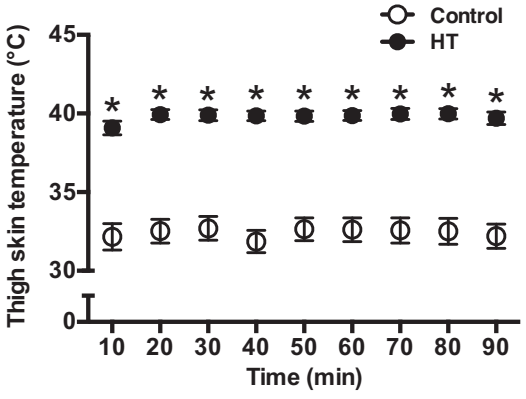


Fig. 1. Thigh skin temperature during exposure to 90 min of heat therapy (HT) or the control intervention. Data were analyzed with a 2-way repeated-measures ANOVA. Values are means ± SD. *$P < 0.05$ vs. control.

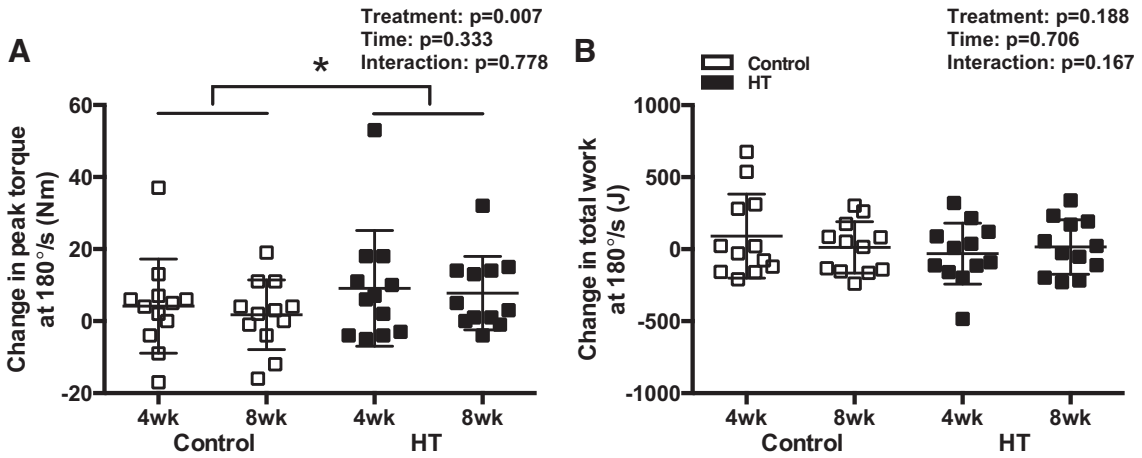


Fig. 2. Individual and group mean changes from baseline in muscle strength (*A*) and fatigue resistance (*B*) following 4 and 8 wk of heat therapy (HT) or the control intervention. Data were analyzed with a 2-way repeated-measures ANOVA. *Main effect of treatment ($P < 0.05$).

repeated-measures ANOVA was employed to compare the changes from baseline in all variables between the leg exposed to HT and the control leg. A Tukey post hoc analysis was performed when appropriate. For all analyses, $P < 0.05$ was considered statistically significant.

RESULTS

Thigh skin temperature. Figure 1 displays the temporal profile of thigh skin temperature during exposure to 90 min of HT or the control regimen. The average temperature in the thigh assigned to receive HT was 39.8 ± 0.3°C, whereas in the control leg the average temperature was 32.4 ± 0.3°C (main treatment effect, $P < 0.001$).

Muscle strength and fatigability. In the thigh that received HT, maximal isokinetic peak torque of the knee extensors at 180°/s improved by 6% at *week 4* and by 5% at *week 8* (baseline: 140 ± 40 Nm, 4 wk: 149 ± 50 Nm, 8 wk: 148 ± 46 Nm) (Fig. 2). Conversely, in the control thigh peak torque increased by 2% and 1% at *weeks 4* and *8*, respectively (baseline: 142 ± 43 Nm, 4 wk: 147 ± 44 Nm, 8 wk: 144 ± 45 Nm). Comparison of the changes from baseline in peak torque revealed a significant main effect of treatment ($P = 0.007$) but no time effect ($P = 0.333$) or treatment × time interaction ($P = 0.778$). Fatigability, as assessed by the total work completed during 40 consecutive maximal contractions at 180°/s, was not altered either after exposure to HT (baseline: 4,434 ± 1,232 J, 4 wk: 4,404 ± 1,310 J, 8 wk: 4,449 ± 1,281 J) or in the control thigh (baseline: 4,309 ± 1,122 J, 4 wk: 4,400 ± 1,286 J, 8 wk: 4,321 ± 1,153 J) (Fig. 2).

Fiber type distribution and morphology. Muscle fiber cross-sectional area (CSA), perimeter, SF, as well as fiber type distribution are shown in Table 1. There were no treatment, time, or treatment × time effects for the changes in fiber CSA in both fiber types. Fiber type distribution was also not significantly influenced by HT.

Capillarization. The number of capillary contacts in type I fibers declined throughout the study in both the control thigh (baseline: 5.0 ± 1.1, 4 wk: 4.7 ± 0.8, 8 wk: 4.8 ± 0.8) and the thigh treated with HT (baseline: 5.1 ± 1.0, 4 wk: 4.7 ± 0.8, 8 wk: 4.7 ± 0.8) (Fig. 3). Exposure to HT also had no significant effect on other capillarization indexes in type I fibers (Fig. 3). Conversely, although the number of capillary contacts around type II fibers declined by nearly 10% in the control thigh (baseline: 4.6 ± 0.6, 4 wk: 4.2 ± 0.7, 8 wk: 4.2 ± 0.5), exposure to HT prevented a temporal reduction in this variable (baseline: 4.2 ± 0.6, 4 wk: 4.2 ± 0.6, 8 wk: 4.3 ± 0.8). A significant treatment effect was observed for the changes in CC ($P = 0.016$), C:Fi on an individual fiber basis ($P = 0.007$), and CFPE ($P < 0.001$) in type II fibers (Fig. 3).

Mitochondrial content. The changes in maximal CS activity and the content of OXPHOS protein complexes are shown in Table 2. There was no treatment or time effect or treatment × time interaction for levels of the mitochondrial OXPHOS proteins measured.

Table 1. *Muscle fiber morphological measurements*

	Control			Heat Therapy		
	Week 0	Change at *week 4*	Change at *week 8*	*Week 0*	Change at *week 4*	Change at *week 8*
Type I CSA, μm^2	6,013.3 ± 1,136.4	−20.3 ± 1,202.2	−533.5 ± 1,193.2	6,394.2 ± 1,608.9	−431.9 ± 1,717.7	−315.5 ± 2,234.2
Type II CSA, μm^2	7,186.8 ± 1,168.2	−443.9 ± 1,638.6	−1,192.3 ± 1,958.7	6,960.6 ± 1,208.1	−488.6 ± 2,141.1	−564.3 ± 1,934.0
Type I perimeter, μm	326.2 ± 28.1	7.3 ± 34.0	−11.7 ± 33.5	339.2 ± 42.7	−18.4 ± 37.5	−6.2 ± 67.7
Type II perimeter, μm	367.4 ± 60.9	−14.2 ± 59.9	−35.7 ± 67.9	359.0 ± 35.7	−23.7 ± 44.9	−17.8 ± 62.6
Type I SF	2.53 ± 0.2	−0.06 ± 0.3	0.07 ± 0.2	2.62 ± 0.1	0.02 ± 0.1	−0.10 ± 0.2
Type II SF	2.58 ± 0.2	−0.03 ± 0.3	0.05 ± 0.2	2.59 ± 0.2	0.05 ± 0.2	−0.06 ± 0.3
Type I, %	38.4 ± 6.1	−2.01 ± 8.9	−1.45 ± 5.3	42.5 ± 14.0	−3.82 ± 9.0	−2.73 ± 12.2
Type II, %	61.6 ± 6.1	2.01 ± 8.9	1.45 ± 5.3	57.5 ± 14.0	3.82 ± 9.0	2.73 ± 12.2

Values are means ± SD. CSA, cross-sectional area; SF, sharing factor; *week 0*, baseline values before treatments; Change at *week 4* and Change at *week 8*, changes from baseline value after 4 and 8 wk of heat therapy or control intervention.

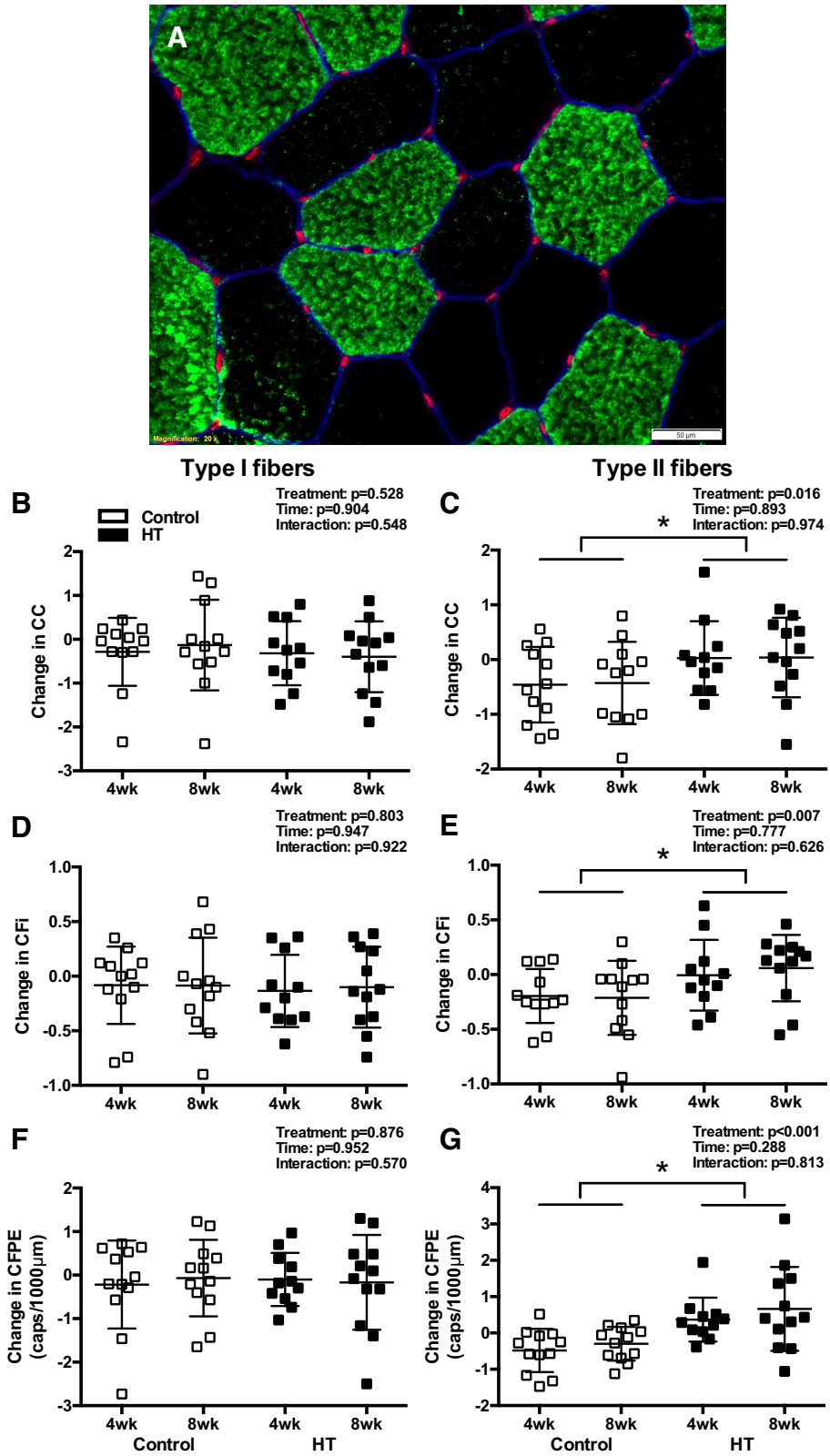


Fig. 3. *A*: representative skeletal muscle cross section displaying immunoreactivity for dystrophin (blue), CD31 (red), and myosin heavy chain type I (green). *B* and *C*: changes from baseline in the number of capillary contacts (CC) for type I (*B*) and type II (*C*) fibers. *D* and *E*: changes from baseline in the ratio of the number of capillaries to each muscle fiber (C:Fi) for type I (*D*) and type II (*E*) fibers. *F* and *G*: changes from baseline in the capillary-to-fiber perimeter exchange index (CFPE) for type I (*F*) and type II (*G*) fibers. Data were analyzed with a 2-way repeated-measures ANOVA. *Main effect of treatment ($P < 0.05$).

Expression of angiogenic factors and heat shock proteins. A main effect of treatment was observed for the changes in skeletal muscle eNOS content ($P = 0.003$), whereas eNOS^{ser1177} phosphorylation ($P = 0.389$) and eNOS phosphorylation normalized to eNOS content ($P = 0.201$) were not altered by the intervention (Fig. 4). The protein content of members of the small heat shock protein (HSP20) family, alphaB-crystallin (HSPB5) (main effect of treatment, $P =$

Table 2. *Changes in maximal citrate synthase activity and content of OXPHOS protein complexes*

	Control		Heat Therapy	
	Week 4	*Week 8*	*Week 4*	*Week 8*
Maximal citrate synthase activity, nmol·min^{-1}·mg protein^{-1}	−4.40 ± 14.43	2.04 ± 11.43	−0.84 ± 6.83	−2.94 ± 14.05
Fold changes in OXPHOS protein complexes				
Complex I	1.03 ± 0.35	1.11 ± 0.54	0.98 ± 0.26	1.08 ± 0.33
Complex II	0.89 ± 0.30	0.96 ± 0.38	0.90 ± 0.28	0.93 ± 0.33
Complex III	1.06 ± 0.15	1.06 ± 0.21	1.02 ± 0.11	1.03 ± 0.13
Complex IV	1.02 ± 0.36	1.10 ± 0.46	0.92 ± 0.23	0.97 ± 0.44
Complex V	1.01 ± 0.17	1.02 ± 0.22	0.99 ± 0.15	1.01 ± 0.18

Values are means ± SD. OXPHOS, oxidative phosphorylation; *week 4* and *week 8*, changes from baseline value after 4 and 8 wk of heat therapy or control intervention.

0.007) and heat shock protein family B member 1 (HSPB1) (main effect of treatment, $P = 0.009$), was also significantly higher in the thigh treated with HT (Fig. 4). No treatment effect was observed for the changes in VEGF, ANGPT1, HSPA1A and the HSP90 family members (Fig. 4).

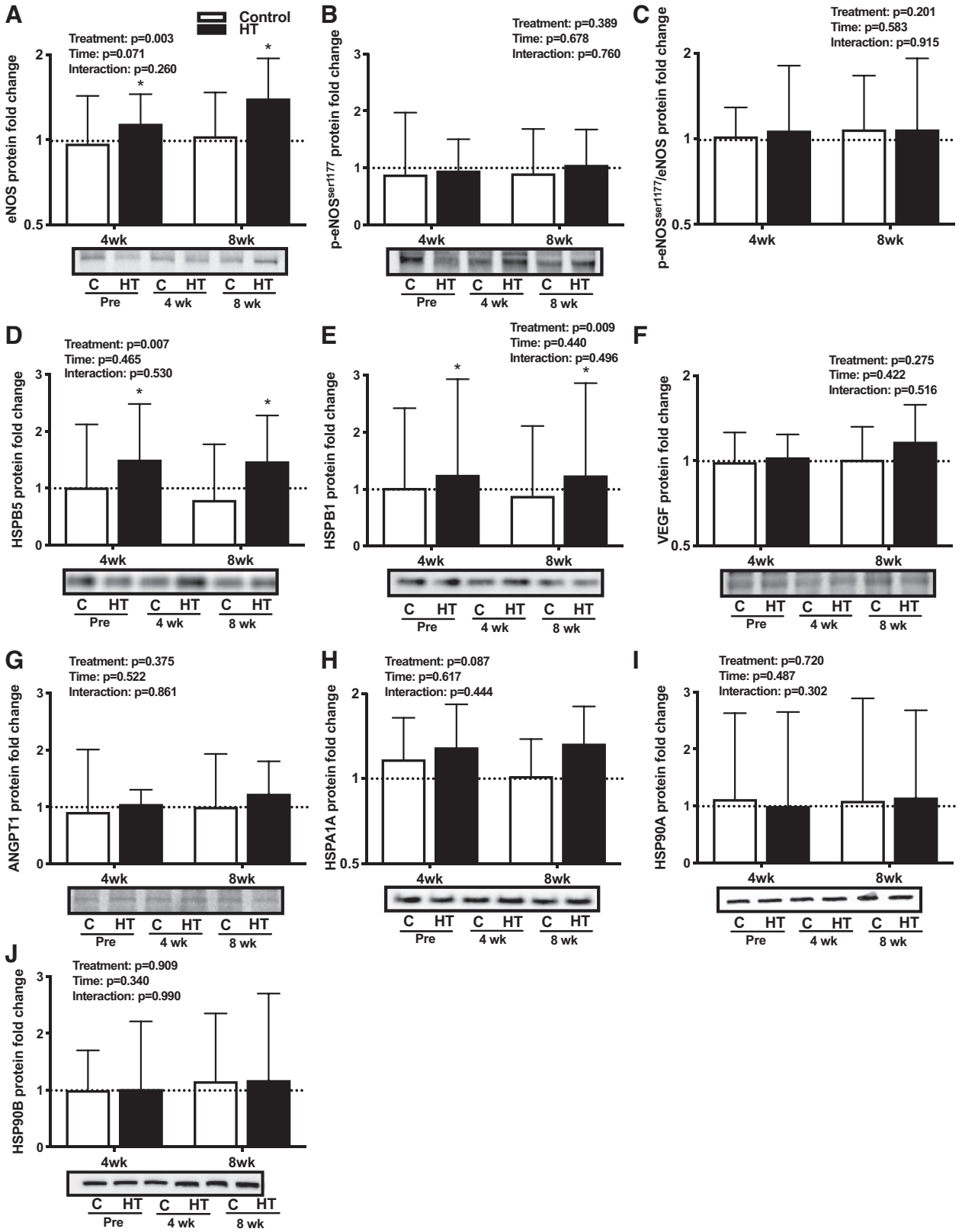
DISCUSSION

The primary findings of this study were that repeated local thigh heating for 8 wk elicited an increase in eNOS content and averted a temporal decline in skeletal muscle capillarization indexes. Conversely, HT had no effect on skeletal muscle mitochondrial content. Confirming earlier observations that exposure to local and whole body heat stress improves skeletal muscle contractile function (13, 32), we also report that 8 wk of local HT enhanced the strength of the knee extensors. Combined, these findings indicate that a simple and well-tolerated HT modality significantly influences skeletal muscle morphology and function and sheds new light on the potential therapeutic use of local heat stress to treat conditions associated with skeletal muscle abnormalities.

Experimental considerations. We chose to apply local HT for 90 min in each session in the present study because we previously showed that this regimen elicits increased expression of heat shock proteins and angiogenic mediators in human skeletal muscle (25). Thus participants were required to spend 90 min daily (5 days/wk) sitting in the laboratory to receive HT and control treatments. One unintended consequence of this demanding protocol was that some participants reported being unable to maintain their habitual exercise routines throughout the study because of time constraints. Although we did not directly measure physical activity patterns, analysis of weekly reports by the participants revealed that seven individuals had marked decrements in exercise time throughout the study and three others reported modest changes. The reduction in structured physical activity coupled with increased sedentary time might be partially responsible for the observed small, albeit consistent, decline in fiber CSA (Table 1) and capillarization (Fig. 3), particularly in the thigh assigned to the control regimen. Several studies have shown that short periods of reduced physical activity (e.g., step reduction) impair glucose metabolism, including insulin sensitivity (24), and lower myofibrillar protein synthesis rates (33) in healthy young adults. More severe forms of muscle disuse, such as 2 wk of single-leg limb immobilization, lead to reduced leg lean mass and muscle capillarization in old and young men (39). Of note, exposure to HT has been shown to attenuate the manifestations of skeletal muscle disuse in animals (37) as well as in humans (15). Our findings that daily local HT prevented the decline and/or enhanced indexes of capillarization (Fig. 3) relative to the control intervention add to this growing body of literature that indicates that HT mitigates the detrimental consequences of physical inactivity in skeletal muscle.

Effect of HT on muscle capillarization. The ability of heat stress to promote a proangiogenic milieu in skeletal muscle and a consequent increase in capillarization was first documented by Akasaki and coworkers in a model of peripheral arterial insufficiency (1). These authors showed that mice treated with far-infrared dry sauna daily for 5 wk had greater capillary density and eNOS expression in the ischemic muscle. Of note, chronic treatment with the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester abolished the changes in capillarization as well as the recovery in blood flow (1). Similarly, the angiogenic response to heat stress was absent in mice lacking eNOS (1). Recently, these earlier observations in ischemic mouse skeletal muscle were extended to humans. Hesketh and coworkers reported that 6 wk of whole body passive HT increased capillary density by 21% and endothelium-specific eNOS content by 8% in the vastus lateralis muscle of sedentary young individuals (19). The increase in eNOS content and the consequent angiogenic response to whole body HT appear to be mediated in part by circulating factors. Brunt and coworkers showed that exposing cultured endothelial cells to serum collected from participants who had undergone whole body HT for 8 wk increased the abundance of eNOS and endothelial tubule formation (6). Combined, these studies provide compelling evidence implicating nitric oxide as a critical mediator of heat-induced skeletal muscle angiogenesis.

In light of these earlier reports, we examined the effects of local HT on the content of eNOS and muscle capillarization. In accordance with the previous findings from whole body heating (19), we report that eNOS content was 18% and 35% higher in the thigh exposed to HT compared with the control thigh at 4 and 8 wk, respectively (Fig. 4). Changes in eNOS were accompanied by significant differences in capillarization between HT and control in type II but not type I fibers (Fig. 3). The mechanistic basis underlying the fiber type-specific effect of HT on capillarization is unclear. Increased wall shear stress in the capillary network has been proposed to be a critical signal for promoting HT-induced skeletal muscle angiogenesis (1, 19). Studies in animals (2) as well in humans (17) have documented a modest increase in muscle blood flow during exposure to local heat stress. It is possible to speculate that type II fibers experienced a greater relative increase in blood flow (and wall shear stress) during HT compared with type I fibers.



Alternatively, it is possible that the effects of HT were mostly evident in type II fibers because capillarization around these fibers was more severely impacted by reduced physical activity levels (Fig. 3). Of note, Hesketh and coworkers did not observe differences between fiber types in the magnitude of the increase in capillarization following 6 wk of whole body HT (19).

Contrary to our hypothesis, we did not observe changes in the content of VEGF and ANGPT1 levels after treatment with local HT. We previously reported that the expression of these pivotal angiogenic mediators is enhanced after a single session (25) as well as 5 days of repeated exposure to HT in injured muscle (22). It is plausible that the levels of these factors were temporarily increased early in the intervention period and later declined toward baseline levels. A similar scenario might explain the lack of effect of local HT on the content of several members of the heat shock protein family, including HSP70 and HSP90. One important exception was the marked increase in the content of small heat shock proteins HSPB5 and HSPB1 in the thigh exposed to HT (Fig. 4). This is an important observation because small heat shock proteins have been implicated in the regulation of angiogenesis and blood vessel function in multiple tissues (10, 21). Additional studies are warranted to define the role these molecular chaperones exert in heat-induced skeletal muscle angiogenesis.

Heat stress and mitochondrial biogenesis. The finding that heat stress induces mitochondrial biogenesis in C2C12 myotubes (27) has led to several investigations asking whether repeated HT could potentially enhance mitochondrial content in vivo. Experiments in mice revealed that daily exposure to whole body heat stress (5 days/wk for 3 wk) increased mitochondrial enzyme activities and respiratory chain protein content in skeletal muscle (38). More recently, local heating of the vastus lateralis for 6 consecutive days (2 h daily) increased mitochondrial respiratory capacity and mitochondrial content (16). In contrast, we did not observe a significant effect of local HT on the content of respiratory chain proteins or maximal CS activity in the present study. Our findings align closely with the recent report of Hesketh and coworkers that repeated whole body HT had no effect on skeletal muscle mitochondrial density despite marked effects on exercise capacity and capillarization (19). The inconsistent effect of HT on mitochondrial content may be partially explained by variations in the magnitude and duration of heat stress as well as the modality used for heat induction in skeletal muscle. Pulsed short-wave diathermy, which produces rapid and marked deep tissue heating (12), may be more effective at producing mitochondrial adaptations than superficial heat modalities as employed in the present study. It is worth noting that we have not measured the content of AMP-activated protein kinase (AMPK), peroxisome proliferator-activated receptor gamma, coactivator-1 alpha (PGC1a), and other biomarkers of mitochondrial biogenesis nor assessed the effects of HT on mitochondrial respiration. Hafen and coworkers showed that despite the lack of changes in CS activity, a common surrogate marker of mitochondrial content, local HT using diathermy increased the content of PGC1a and the phosphorylation of AMPK and resulted in improved mitochondrial respiratory capacity (16).

HT and skeletal muscle strength. Given that local heat treatment of the thigh for 8 h/day for 10 wk improved maximal isometric force in young individuals (13), we questioned whether 90 min of thigh heating over 8 wk would significantly enhance knee extensor strength. In agreement with the findings of Goto and coworkers (13), maximal isokinetic torque increased to a greater extent in the thigh exposed to HT compared with the control thigh with just 90 min of treatment (Fig. 2). This improvement in force after treatment with local HT occurred despite the lack of significant differences in fiber CSA between treatments (Table 1), indicating that adaptations other than changes in fiber size explain the observed improvements in force-generating capacity. Of note, Racinais and colleagues demonstrated that as little as 11 days of whole body heat stress increased peak twitch amplitude and torque production of the plantar flexors in humans (32). As it seems unlikely that major changes in fiber size would occur in this short period of time, these findings imply that alternative mechanisms, including increases in force per cross bridge or possibly the kinetics of formation of cross bridges, contribute to strength gains to heat therapy (32). Of note, the study of Racinais et al. did not include a sham-treated group, and it is thus impossible to exclude the possibility that the improvement in muscle function derived partially from a time and/or familiarization effect (32). Further research is needed to explore the mechanistic basis of enhanced force-generating capacity of muscles exposed to repeated heat stress. Additional studies are also warranted to define whether, in addition to muscle strength, HT may affect muscle power and improve performance during submaximal, prolonged events.

Limitations. An important limitation of the present study is that we have not directly measured intramuscular temperature during exposure to local HT. Studies that employed water-circulating garments perfused with warm water to heat the calf or the entire leg of healthy individuals reported average increases in intramuscular temperature ranging from 2.5 to 4°C (8, 17). As we utilized a similar heating modality and treatment regimen, it is tempting to suggest that comparable changes in temperature occurred in the present study. Nonetheless, it is worth highlighting that the time course and magnitude of changes in muscle temperature upon exposure to heat treatment may be modulated by a number of factors, including the treatment duration and the thickness of the subcutaneous fat layer (31).

Another limitation that is inherent to HT studies is the fact that participants cannot be blinded to the intervention. This imposes a challenge for the interpretation of experimental

Fig. 4. Fold changes in skeletal muscle protein expression relative to the baseline sample of select stress management and angiogenic proteins. *A*: endothelial nitric oxide synthase (eNOS). *B*: eNOS phosphorylated at Ser1177 (p-eNOS^{ser1177}). *C*: ratio of p-eNOS^{ser1177} to eNOS. *D*: alphaB-crystallin protein (HSPB5). *E*: heat shock protein family B member 1 (HSPB1). *F*: vascular endothelial growth factor (VEGF). *G*: angiopoietin 1 (ANPTT1). *H*: 72-kDa heat shock protein (HSPA1A). *I*: 90-kDa heat shock protein alpha class A member 1 (HSP90A). *J*: 90-kDa heat shock protein alpha class B member 1 (HSP90B). The baseline sample was assigned a value of 1 and is represented by the dashed line. Data exhibiting skewed distribution (HSPB5, HSPB1, p-eNOS, ANGPT1, HSP90A, HSP90B) were log-transformed before statistical analysis. Values are means ± SD or geometric mean × / ÷ geometric standard error if the variable value was log-transformed. C, control; HT, heat therapy. Data were analyzed with a 2-way repeated-measures ANOVA. *Main effect of treatment ($P < 0.05$).

outcomes that are prone to the placebo effect, including voluntary force production. It is plausible that the observed increase in muscle strength after treatment with HT may be partially ascribed to a placebo effect. This seems unlikely given the accumulating evidence derived from animal studies that repeated that heat stress enhances muscle strength and prevents disuse-induced muscle weakness. For instance, we recently reported that repeated immersion in a water bath at 37°C and 39°C for 3 wk enhanced maximal absolute force of the soleus muscle in a model of ischemia-induced muscle damage (23). Similarly, Yoshihara and colleagues reported that 3 days of whole body HT using a heat chamber (40–41°C for 60 min) abrogated ventilator-induced diaphragm contractile dysfunction in rats (40). These findings reveal that repeated heat stress elicits adaptations that culminate in an improved force generation capacity. Nonetheless, it is imperative that future studies in humans compare the effects of HT on muscle force with a placebo rather than a control intervention (3).

Clinical implications. Water-circulating garments are amenable for home use, do not require supervision by a therapist, and are practical for individuals with restricted locomotion who cannot participate in exercise (e.g., severe peripheral artery insufficiency, chronic heart failure, and chronic obstructive pulmonary disease). Our findings that local HT enhances muscle strength and affects muscle capillarization indicate that this method may be a feasible tool to treat these chronic conditions. One caveat regarding the clinical use of tube-lined garments for HT is that this modality is designed primarily to manipulate skin temperature (9). Prolonged exposure to this method is necessary to attain significant increases in intramuscular temperature (8). For example, 1 h of perfusion of 50°C water through a garment covering a single leg raised the vastus lateralis muscle temperature by ~2.5°C (8). Substantially faster and greater increases in intramuscular temperatures can be achieved with the use of deep tissue heating modalities, such as short-wave diathermy. Garrett and coworkers showed that diathermy application for 20 min raised triceps surae muscle temperature by ~3.5°C (12). Deep tissue heating modalities may therefore possibly confer benefits that are similar or superior to those reported here despite a substantially lower treatment duration. It should be emphasized, nonetheless, that diathermy is less accessible than superficial heating modalities because these devices are expensive and cumbersome and require a trained professional for proper operation.

In addition to its use in rehabilitation, there is evidence that HT may be an ergogenic aid to boost the adaptations to exercise training. For example, Tamura and coworkers recently showed that postexercise whole body heat stress (40°C, 30 min/day, 5 days/wk, 3 wk) additively enhanced endurance training-induced mitochondrial adaptations in mouse skeletal muscle (38). Goto and coworkers showed that repeated heating of the elbow flexor muscles with a heating and steam-generating sheet before and during low-load resistance exercise resulted in greater changes in maximum isometric torque and CSA of the biceps brachii muscle compared with resistance training alone (14). This effect of HT does not appear to occur in the lower limb muscles. Stadnyk and colleagues recently reported that local thigh heating during and for 20 min after resistance exercise of the knee extensors in untrained individuals had no effect on training-induced hypertrophy or function (37). It remains to be determined whether HT may facilitate the adaptations to endurance and resistance training in trained individuals.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

K.K., T.P.G., and B.T.R. conceived and designed research; K.K., B.A.R., C.A.C., B.E.B., B.R., T.P.G., and B.T.R. performed experiments; K.K., B.E.B., Q.S., A.J.T., A.C.P., and B.T.R. analyzed data; K.K., A.J.T., A.C.P., T.P.G., and B.T.R. interpreted results of experiments; K.K. and B.T.R. prepared figures; K.K. and B.T.R. drafted manuscript; K.K., Q.S., A.J.T., A.C.P., S.K., T.P.G., and B.T.R. edited and revised manuscript; K.K., A.J.T., A.C.P., S.K., T.P.G., and B.T.R. approved final version of manuscript.

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