IMPACT OF HEAT THERAPY ON SKELETAL MUSCLE STRUCTURE AND FUNCTION

by

Kyoungrae Kim

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THE PURDUE UNIVERSITY GRADUATE SCHOOL
STATEMENT OF COMMITTEE APPROVAL

Dr. Bruno T. Roseguini, Chair
Department of Health and Kinesiology

Dr. Timothy P. Gavin
Department of Health and Kinesiology

Dr. Chad C. Carroll
Department of Health and Kinesiology

Dr. Shihuan Kuang
Department of Animal Science

Approved by:
Dr. David B. Klenosky

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ABSTRACT

Skeletal muscle occupies approximately 40 to 50 percent of body mass and is responsible for respiration, postural control, and locomotion and plays a pivotal role in regulating glucose, lipid, and protein metabolism. Acute muscle trauma and chronic disease conditions such as muscular dystrophies are associated with structural abnormalities, enhanced fatigability and impaired metabolism and consequently lead to exercise intolerance and poor quality of life. Despite the clinical importance and a number of studies on the treatment of muscle damage, few modalities have shown to elicit beneficial effects. Heat treatment has been used for a long time to treat soft tissue injuries in the field of physical therapy and sports medicine. However, the underlying mechanisms by which heat treatment accelerates muscle recovery following injury are not clear.

The primary aim of my dissertation studies was to determine the impact of heat therapy on skeletal muscle structure and function in humans and animals. In Chapter 2, we report that a single session of local heat treatment promotes the expression of angiogenic and myogeneic mediators including vascular endothelial growth factor (VEGF) and angiopoietin 1(ANGPT1) in healthy human skeletal muscle. In Chapter 3, we report repeated exposure to heat therapy stimulates factors involved in muscle repair process and accelerates functional recovery from exercise-induced muscle damage. In Chapter 4, we show that 8 weeks of local heat therapy improves muscle strength of knee extensor and increases skeletal muscle capillarization in type II muscle fibers. In Chapter 5, we describe the effects of heat therapy in a mouse model of ischemia induced-muscle damage. Animals that were exposed to heat therapy at 39°C had improved maximal absolute force and relative muscle mass in the soleus muscle. These observations reveal that the beneficial effects of heat therapy are muscle fiber type specific and dependent on the treatment temperature. In Chapter 6, we review and summarize the outcomes described in Chapters 2-5 and provide a general conclusion as well the clinical implications of our findings.

CHAPTER 1. INTRODUCTION

Physiology of muscle damage

Exercise-induced muscle damage

Acute muscle damage is the most common type of injury occurring during exercise training and sport events. For instance, unaccustomed or high-intense aerobic and resistance exercise, especially activities that involve lengthening (eccentric) contractions, can cause damage to the sarcolemma, contractile proteins and connective tissue. Typical manifestations of exercise-induced muscle damage (EIMD) include strength loss, delayed muscle soreness and muscle protein release (15, 33). These symptoms can be resolved within a few days following minor insults. Following severe injuries, however, these symptoms can last over several weeks and consequently increase the duration of immobilization and risk of further injury (38).

Models of exercise-induced muscle damage

Several experimental models of skeletal muscle damage were introduced to examine causes and mechanisms as well as to develop effective treatment tools. In laboratory models, the morphological and pathological characteristics, such as inflammation, degeneration and regenerations cycles are similar to those observed in acute muscle trauma in humans. Experimental muscle damage models are also useful to investigate the mechanisms of exercise training-induced skeletal muscle adaptations (79).

Due to the ethical issues, human muscle damage induced by direct trauma cannot be replicated in laboratory settings. Instead, current models typically employ repetitive eccentric muscle contractions or whole-body eccentric-biased exercise to promote muscle injury. A number of studies have reported solid evidence of muscle injury following EIMD, such as changes in muscle function and inflammatory markers (14, 61, 67).

Protocols using eccentric exercise normally induce damage to muscle groups that are specifically involved in contractions. In human studies, the model of uniaxial motion such as elbow flexions or knee extensions has been the most widely used. Eccentric exercise bout of single joint movements can be done by using mechanical devices such as an isokinetic dynamometer in which

the range of motion and angular velocity can be precisely regulated or an isotonic instrument in which the external load can be maintained constant during contractions (5, 67). Using these devices, single joint movements can be isolated and the compensatory motion involvement can be minimized throughout the experiment. Unlike whole-body exercise, these models can effectively eliminate the concentric component of muscle contractions. In addition, these models involve solely unilateral action and thus the opposite limb can serve as an unexercised control. On the other hand, eccentric-biased whole-body exercise such as downhill running and downward-stepping utilizes multiple joints and muscle groups (1, 60). Longer-duration and/or greater number of contractions are required to induce comparable injury because the eccentric contraction component is much less intense for most of the whole-body exercise protocol.

The results of circulating level of creatine kinase (CK) and the magnitude of strength loss following eccentric exercise-induced muscle damage revealed that the extent of injury is more extreme in the elbow flexor muscles as compared to the knee extensors. Isometric strength loss (elbow flexor: 58% vs. knee extensor: 24 %) and blood CK level (elbow flexor: 14,000 IU/L vs. knee extensor: 810 IU/L) were greater following eccentric contractions done by the elbow flexors although the number of repetitions was nearly three times higher in the lower extremity models (9, 13, 68, 95). This is probably due to a greater susceptibility of upper extremity muscles, which are less exposed to eccentric actions when compared to lower extremity muscles (15).

Manifestations

Loss of muscle strength

Prolonged loss of muscle strength and/or power is one of the most reliable indirect indicators of muscle damage (15). Recently, Damas and colleagues (19) conducted a large study (n=286) on the indirect markers of EIMD, including maximal voluntary contraction (MVC), soreness (SOR), CK, range of motion (ROM) and muscle circumference (CIR). All the subjects that participated in this study showed significant changes in these indicators for several days following eccentric elbow contractions despite individual differences. They reported significant inverse correlations between the magnitude of the largest decrease in MVC and other proxy markers (SOR, CK, ROM and CIR), thus consolidating MVC as the major indirect indicator of EIMD. Studies using human and animal models also have shown that the magnitude and duration

of strength loss are associated with the severity of morphological abnormalities in skeletal muscle (11, 17).

In general, the magnitude of the decline in muscle strength and the pattern of recovery from muscle damage depend on the mode, novelty and intensity of contractions (15). Compared to the immediate strength loss and fast recovery observed following concentric or isometric contractions, the degree and duration of the functional impairment is much greater following eccentric exercise. Whole-body eccentric exercise protocols such as downhill running typically generate approximately 10-30% strength decline over several days following exercise. A previous study has shown that 45 min of downhill running on the treadmill led to ~25% reduction in muscle strength that persisted for a few days (76). On the other hand, isolated eccentric exercise protocols can generate greater strength loss. For example, maximal eccentric exercise can cause up to 50-65% strength loss following elbow flexions. Rinard and co-workers (75) reported significant force loss (70%) following 70 repetitions of eccentric elbow flexions. In addition, Guilhem and colleagues (25) showed that impaired force production capacity could last longer than 2 weeks following a maximal eccentric exercise bout.

Although the exact mechanisms that induce prolonged loss of strength in EIMD are not precisely established, it has been proposed that popped sarcomeres and excitation-contraction (EC) coupling failure, may be involved (33). Morgan (55) suggested that uneven stretching during repeated lengthening contractions leads to disruption of sarcomeres and ultimately causes a reduction in muscle strength. Several electron microscopy studies later confirmed that eccentric muscle contractions induce cellular and subcellular structural deformations and distortion of the contractile machinery (43). Since overstretched sarcomeres likely generate less force, overload on the membrane and t-tubule components can cause the opening of stretch-activated channels and facilitate calcium influx. In turn, elevated intracellular levels of calcium can activate calcium-dependent proteolytic enzymes, calpains and caspases. Augmented calpain activity can further damage the contractile proteins and calcium regulating proteins, which result in prolonged force deficits (73). Evidence from several animal studies implicates EC coupling failure as an important cause of strength loss. Warren and colleagues (93) reported that there was no difference in force generating capacity between eccentrically damaged and control muscles when these muscles were electrically stimulated in a solution with high calcium concentration. In a follow-up study using an in vivo model, the same research group reported that caffeine restored muscle force when

compared to a control condition. The authors suggested that approximately 50-75% of force loss following exercise-induced muscle damage can be explained by a failure in the E-C coupling process (36).

Delayed onset muscle soreness

Delayed onset muscle soreness (DOMS) is a sensation of local pain or discomfort that usually peaks between 24 and 48 hours after the exercise bout and subsides within a week (33). It is distinguished from neuro-muscular fatigue because DOMS is not noticed until several hours following the exercise trial. Hough (32) described this unique symptom of EIMD for the first time in 1900. He reported that the discomfort lasts several days following repeated finger flexor contractions and the manifestation was obvious only in the untrained finger flexor muscle. It seems to appear again several months later if there were no regular mechanical stress. Despite the extensive reports of DOMS as an indirect marker for EIMD, DOMS does not correlate well with the extent of muscle damage observed on biopsy specimens (62).

A few possible theories have been proposed although the exact mechanisms responsible for the delayed onset muscle soreness following muscle damage are not fully understood. Friden (23) reported increased muscle fiber size and intramuscular fluid pressure in the tibialis anterior muscle 48 hours after repetitive eccentric muscle contractions suggesting a causal relation between fiber swelling and DOMS. Crenshaw (18) later confirmed this notion by reporting that soreness is associated with changes in intramuscular fluid pressure of the vastus lateralis muscle at rest and during movement. Increased fiber swelling and activated free nerve endings located in muscle fibers may contribute to the sensation of muscle soreness (61). Noxious chemicals released during inflammation also have been known to produce the perception of soreness, which is mediated by type III and IV afferent neurons (63). A human study indeed confirmed that intramuscular injection of algogenic substances into tibialis anterior muscle results in hyperalgesia (4). Bradykinins and prostaglandins seem to act directly on muscle nociceptors or promote the expression of proteins such as nerve growth factor, which acts on the nociceptors.

Circulating creatine kinase

Appearance of muscle-specific proteins in the blood is an indirect marker of muscle damage and impaired function of the sarcolemma, which supposedly is not permeable to

intramuscular enzyme proteins such as creatine kinase (CK), lactate dehydrogenase, and myoglobin. Compared to the other muscle proteins, CK has received the most attention due to the greater magnitude of increase and low cost of the analysis (15). It is unclear whether the increased membrane permeabilization is caused by direct disruption of the sarcolemma or by stretch-activated Ca^{2+} activity, but the stress-susceptible structure of the extracellular matrix (ECM) is partly responsible for sarcolemmal dysfunction (33).

Structural damage

Exercise-induced muscle damage initiates with focal structural damage such as deformation of Z-disks and desmin in the myofibrils by excessive mechanical stress primarily at the site of myotendinous junctions (88). In addition, eccentric contractions can result in disruption of the sarcolemma, which is important to maintain low calcium concentrations in the cytoplasm by regulating calcium permeability or calcium-ATPase pump activity. Calpains and caspases are well known calcium-dependent cysteine and cysteine-aspartic proteases capable of actomyosin destruction. Their activity dramatically increases when Ca^{2+} ions accumulates in the cytoplasm. Raastad and coworkers reported, for the first time, increased calpain activity in human muscle after EIMD (73). They showed that an excessive amount of eccentric exercise (300 contractions in one leg, no contraction in another leg) significantly increased calpain activity compared with control condition. Until 7 days after the muscle damage, calpain activity remained at a higher level than control and the capacity of force development was not fully restored. In addition, they observed myofibrillar disruptions in ~36 % of all fibers in exercise muscle.

Ischemia-induced muscle damage

Patients with chronic diseases such as peripheral artery disease (PAD) have insufficient blood perfusion to the lower extremities, which leads to ischemia-induced muscle damage. Results obtained from light microscopy evaluation and immunohistochemical staining of muscle biopsy specimens provided strong evidence of myopathic changes in skeletal muscle in patients with PAD, such as necrotic fibers and fibrosis. Such changes are also associated with neuropathic changes as evidenced by significant myofibrillar denervation (28, 50, 51)

Models of ischemia-induced muscle damage

Although progressive atherosclerosis is the major cause of peripheral artery disease in human patients, acute limb ischemia caused by ligation and/or excision of the femoral artery has been the most commonly employed strategy to simulate PAD in preclinical models (49, 94). Several animal models have been used to investigate mechanisms and/or therapeutic modalities that mediate revascularization of ischemic muscle, but the magnitude of post-ischemic injury outcomes vary substantially between models (78). Following immediate limb ischemia, C57BL/6J mice have faster perfusion and functional recovery when compared to BALB/cJ mice. These differences are caused in part by different genetic background such as preexisting vascular anatomy and capacity for post-ischemic vascular remodeling. For example, C57/BL6 mice had the greatest collateral growth and almost no changes in capillarization while BALB/cJ mice showed greatest increase in capillarization but modest changes in collateral development (69).

Manifestations

Exercise intolerance

Patients with peripheral artery disease have severe strength loss and reduced walking capacity, which lead to not only poor quality of life but also high risk of morbidity and mortality (31). Reduced blood flow to the muscle tissue seem to be the primary factor that limits exercise performance in PAD patients. However, accumulating evidence indicates that altered skeletal muscle structure and function may also contribute to the impairment in exercise tolerance in PAD patients (71). In addition, calf muscle mass is approximately 15% lower in PAD patients as compared with their healthy counterparts.

Endothelial dysfunction & revascularization

Patients with hindlimb ischemia have significantly impaired endothelial function. For example, increased arterial stiffness, oxidative stress and elevated levels of the potent vasoconstrictor endothelin-1 negatively affect exercise-induced vasodilation and consequently limit exercise tolerance in these patients (29). Following an arterial occlusion, compensatory mechanisms are activated including arteriogenesis, the growth of pre-existent arterioles, and angiogenesis, new capillary growth (90). Collateral growth mainly involves cell proliferation, which contributes to a marked increase in vessel length and diameter. Although the mechanisms

mediating the collateral vessel enlargement after arterial occlusion are unclear, the sudden increase in blood flow through the pre-existing arterioles and augmented fluid shear stress seem to be of primary importance (16). Increased blood flow velocity and viscous drag in collaterals stimulate endothelial nitric oxide synthase (eNOS), nitric oxide (NO) release and secretion of monocyte chemoattractant protein-1 (MCP-1) by the smooth muscle cells. Results from animal studies highlight the important role of monocytes in collateral growth. Increased accumulation of monocytes in the smooth muscle cells and augmented activities that include high expression of vascular growth factors like VEGF and FGF2 promote enlargement of pre-existing arterioles (90).

Myopathy and mitochondrial dysfunction

The myopathy is readily observed in histologic preparations of gastrocnemius muscle following ischemia-induced muscle damage. Human studies using gastrocnemius muscle biopsy specimens from PAD patients reported significantly altered myofiber size and appearance. Limb ischemia causes impaired blood flow and tissue hypoxia, which leads to tissue necrosis. The pathological process of ischemia-induced muscle damage includes myofibre necrosis, phagocytocis, fibrosis as well as significant reduction in myofibrillar innervation (70).

Mitochondria carry out several critical tasks such as energy metabolism (aerobic and anaerobic metabolism) and participate in intercellular communication and cell-death pathways (redox signaling and apoptotic process). Examination of mitochondria from PAD muscle revealed both quantitative and qualitative ultrastructural abnormalities as well as impaired energy metabolism (carbohydrate and acyl-CoA oxidation). Further investigations in animal models demonstrated significant reductions in enzymatic activities and mitochondrial respiration in mitochondrial electron transport chain complexes (48).

Physiology of muscle repair

Skeletal muscle repair is complex and involves inflammation, myofiber degeneration and regeneration, angiogenesis and ECM remodeling.

Satellite Cells and myofiber regeneration

Skeletal muscle has a remarkable capacity for growth and repair. Satellite cells or myogenic precursor cells (MPCs) play critical roles during post-mitotic adult skeletal muscle

repair and maintenance. Satellite cells reside between basal lamina and sarcolemma and are inactive in the resting condition. Upon muscle damage, satellite cells exit from quiescence and proliferate; some of daughter cells continue to differentiate whereas others return to the quiescent condition to replenish the reservoir population of satellite cells in case of future events (42). Satellite cells committed to myogenesis can be added into the regenerating fibers or fuse together to form new myotubes (central nucleation) and undergo terminal differentiation and maturation (peripheral nucleation) (92).

Each stage of muscle regeneration is regulated by muscle-specific transcription factors including myoblast determination protein (MyoD), myogenin, and myogenic factor 5 (Myf5). For instance, Myf 5 and MyoD allow the myogenic satellite cells to continue muscle lineage while myogenein appears to be associated with promotion of muscle differentiation. Increased myocyte enhancer factor-2 (MEF2) expression also promotes fusion and transition to terminal differentiation in which muscle-specific genes such as myosin heavy chain (MHC) maintain high levels of expression. A reduction or depletion of the satellite cell pool negatively impacts muscle repair. For example, satellite cell deficient mice have markedly impaired regenerative capacity and excessive collagen infiltration following cardiotoxin-induced muscle injury (58). Multiple factors including aging (74) and the magnitude and type of stimulation (34) can affect satellite cell function and activity, but inflammation likely plays a dominant role during muscle regeneration (12). Following an acute trauma or EIMD, inflammatory cells interact with satellite cells, endothelium and stromal cells and tightly regulate the tissue repair process for adequate recovery.

Inflammation and myofiber regeneration

Once skeletal muscle is injured, a series of inflammatory reactions begin. During the initial phase of inflammation, called pro-inflammation, circulating myeloid cells migrate into the injured site and infiltrate into the perimysium and endomysium with chemotactic assistance (88). Among several immune cell types, neutrophils and macrophages are known to play important roles during the initial inflammation. These inflammatory cells clear debris from the injured area in preparation for muscle regeneration. They secrete several cytokines and growth factors that are associated with the activation and proliferation of muscle progenitor cells. In the later phase of inflammation,

called anti-inflammation, the immune cells release anti-inflammatory cytokines that promote satellite cell fusion, larger myotube formation and growth.

Neutrophils contribute to the regeneration process in two ways, through phagocytic function and by releasing inflammatory cytokines, both of which are important steps in the early phase of muscle repair. However, the action of these cells can lead to secondary damage to the healthy surrounding tissue (82). For instance, neutrophil depletion prior to exercise-induced muscle injury reduced muscle damage in animal models (40, 72). Whereas neutrophils are primarily important in the early phase of regeneration, macrophages participate in the entire muscle repair process (12).

M1 macrophages secrete cytokines such as tumor necrosis factor alpha (TNFα) and interferon gamma (INFγ) both of which stimulate the activation and proliferation of satellite cells during this initial phase of muscle repair (77, 88). A few days later, M1 macrophages change their phenotype to acquire anti-inflammatory profiles that release anti-inflammatory cytokines (2). M2 macrophages secrete interleukin 10 (IL-10) and insulin-like growth factor 1 (IGF-1), which stimulate differentiation of satellite cells, fusion into a large myotube, and growth toward mature myofibers. Transforming growth factor beta 1 ($TGF_{\beta}1$) released by M2 macrophages also plays an important role in ECM remodeling by stimulating the differentiation and maturation of fibro-adipogenic precursors (FAPs). Interleukin 4 (IL-4) expressed by eosinophils also contributes to enhancing ECM remodeling by proliferating FAPs. Recently, TNFα and M1 macrophages have shown to mediate ECM remodeling through apoptosis and phagocytosis of FAPs during the pro-inflammatory stage.

Efficient transition between M1 and M2 macrophage phenotypes seems to be required for proper muscle regeneration because these two different types of macrophages orchestrate the pro- and anti-inflammatory stages and influence satellite and stromal cell function. Studies on the time course of changes in immune cell populations and satellite cell activity revealed that experimental perturbations on the transition from pro-inflammation to anti-inflammation impair the transition between proliferative and differentiation stages of muscle regeneration (89). Accumulating evidence from human and animal studies supports this notion. Takagi and colleagues (2011) tested the influence of icing on sequential changes in allocation of macrophages and satellite cells as well as the expression of transforming growth factor beta 1 (TGF-β1) and insulin-like growth factor 1 (IGF-1), which regulate satellite cell proliferation, differentiation, and regenerating muscle growth

(84). The authors applied icing (0.3~1.3°C) for 20 min on extensor digitorum longus muscle following crush-induced muscle damage and observed chronological changes in inflammation and muscle regeneration up to 24 days. In the icing group, degeneration of necrotic muscle fibers and satellite cell differentiation were delayed approximately 24 hours compared with non-icing group. Along these lines, expression of TGF-β1 and IGF-1, both of which are released by macrophages, was delayed in the icing group. Retarded inflammation in the icing group contributed to immature muscle regeneration and excessive collagen infiltration during muscle remodeling. As excessive collagen deposition inside the sarcolemma was accompanied by impaired contractile muscle function, the authors suggested that cryotherapy should not be used following sport-induced muscle damage. Michailidis (2013) reported that thiol-based antioxidant supplementation after intense eccentric exercise reduced neutrophil and macrophage activities and resulted in blunted pro-inflammatory cytokine release and delayed recovery of contractile function (53). They suggested that the impaired inflammatory response may be related to the attenuated nuclear factor kappa-light-chain-enhancer of activated B cells ($NF\text{-}_kB$) signaling, which controls DNA transcription and is responsible for pro-inflammatory cytokine production and release from muscle or immune cells. Interestingly, the authors also reported that antioxidant supplementation reduced Akt-mTOR-rpS6 signaling, which promotes MyoD-mediated myogenic stem cell differentiation and post-transcriptional protein synthesis. Taken together, these findings highlight the notion that inflammation is necessary for optimal skeletal muscle repair.

Angiogenic signaling and myofiber regeneration

The process of skeletal muscle repair also includes new capillary growth (12). Angiogenesis is a process by which new blood vessels develop. Except cancer cell-derived angiogenesis and wound healing process, new capillary growth mainly takes place in skeletal muscle after endurance exercise training and serve to afford a greater oxygen extraction capacity and better muscle performance (65). There are several known physiological candidates that stimulate angiogenesis in skeletal muscle. Adenosine, a metabolite that accumulates proportionally to the cellular metabolism during exercise, is known to stimulate vascular endothelial growth factor (VEGF) production and endothelium proliferation. Low partial pressure of oxygen ($_PO2$) in the muscle tissue also induces hypoxia inducible transcription factor (HIF)-mediated angiogenesis. Mechanical stimuli including tensional forces, stretch, and shear stress seem to cause angiogenesis

through mechanosensors (cell-cell adhesion proteins and tyrosine receptor kinase) mediated signaling pathway. VEGF plays an important role in angiogenesis after prolonged muscle overload by increasing endothelium proliferation and matrix metalloproteinase 2 (MMP2) and membrane type 1-matrix metalloproteinase (MT1-MMP) contribute to basement membrane degeneration and abluminal sprout formation. In addition, Angiopoietin 1 and 2 (ANGPT1 and ANGPT2) act as strong angiogenic regulators collaborating with VEGF. ANGPT1-dependent activation of Tie2 (tyrosine kinase with immunoglobulin-like and EGF-like domain 2) promotes capillary sprouting (pericytes recruitment and sprout stabilization) while ANGPT2 mediated activation of Tie2 elicits chemotaxis and tube formation in cultured endothelial cells (10, 66).

Several studies have reported the effects of angiogenic mediators on muscle regeneration. Arsic and colleagues reported that VEGF-A delivery using an adeno-associated virus to ischemia-, glycerol-, or cardiotoxin-injured muscle has beneficial effect on skeletal muscle regeneration (3). The expression of tyrosine receptor kinases, VEGF receptor-1 (VEGFR-1) and VEGFR-2, was increased in the satellite cells of the damaged muscle during myotube formation in vitro. Also, Bryan and co-workers (8) showed the important role of VEGF in differentiation and VEGFRs expression in myogenic precursor cells. In that study, VEGF stimulated C2C12 differentiation and myotube hypertrophy while addition of soluble fms-like tyrosine kinase-1 (sFlt1), a VEGF inhibitor, limited myotube hypertrophy and myogenic differentiation. In addition, Deasy and colleagues (20) reported impaired muscle regeneration capacity with significant increase in fibrosis when VEGF activity was inhibited by sFlt1 in Duchene muscular dystrophy (mdx) mice. The effects of ANGPT1 on skeletal muscle regeneration and angiogenesis were also demonstrated by Mofarrahi and colleagues (54). ANGPT1 injection in a model of cardiotoxin-induced muscle injury accelerated recovery 4 days after the injury and improved tibialis anterior muscle regeneration, contractile function and increased capillary density.

Current studies also suggest that the interaction between angiogenic signaling and satellite cell function play an important role during skeletal muscle recovery. Electron microscopy data revealed a high correlation between capillary numbers and satellite cell content (59). Approximately 80% of satellite cells reside within 20 microns from a capillary, suggesting a potential role of interaction between vasculature and satellite cells in muscle homeostasis. Since angiogenesis and myogenesis occur simultaneously, the expression of pro-angiogenic mediators, VEGF and ANGPT1, and their receptors significantly increase after injury and involve in tissue

repair. VEGF has been shown to be expressed by differentiation myogenic cells and regenerating myofibers. As myogenic cells bear the VEGF-R, it also acts by stimulating their migration and protecting them from apoptosis. VEGF may also participate in the myogenic differentiation program through its influence on the myogenic transcription factor MyoD. Indeed, VEGF-null embryonic stem cells show reduced differentiation while VEGF overexpressed cells promote myotube hypertrophy (8). In addition, VEGF delivery using adeno-associated virus markedly improved myofiber reconstitution in ischemia induced muscle damage.

Impact of heat therapy on muscle recovery following damage

As a nonpharmacological intervention, heat therapy has shown to elicit beneficial effects on muscle recovery following musculoskeletal injuries. The mechanisms by which heat therapy works are not firmly established, but accumulating evidence from both human and animal studies indicate that heat stress may have an impact on inflammation, protein turnover, angiogenesis and mitochondrial adaptation.

Effects of heat therapy on inflammation

Several studies investigated the effects of heat therapy on inflammation and muscle regeneration following muscle damage in animal models. Takeuchi and colleagues (85) examined the impact of heat stress on macrophage activity and satellite cell content as well as the expression of transforming growth factor beta 1 (TGF-β1) and insulin-like growth factor 1 (IGF-1), which mediate satellite function and fate. The authors applied a hot pack (42°C) for 20 min on the extensor digitorum longus muscle following crush injury in rats and examined the changes in inflammation and muscle regeneration over 24 days. In the heat muscle, degeneration of necrotic muscle fibers was accelerated in response to increased migration of macrophages in the early phase of muscle regeneration. These findings were associated with accelerated satellite cell activation and improved muscle regeneration. The authors pointed out that heat therapy may accelerate macrophage accumulation at the beginning of the remodeling process and optimize the timing of consecutive inflammatory actions. Based on these previous findings, Shibaguchi and co-workers (80) examined the effects of repeated heat (42°C, 30 min, 2 – 14 days, and every other day) or ice (0°C, 20 min soon after the injury) after bupivacaine induced muscle injury. In agreement with other studies, intermittent heat stress facilitated muscle mass restoration toward the control level,

increased satellite cell proliferation in the Th1 inflammatory phase, and inhibited muscle fibrosis. The authors observed dramatically decreased heat shock protein 72kDa (HSP72) expression, which plays a role in protecting cells against cellular stress, in all muscle damage groups after bupivacaine induced tissue damage compared to the control group, but HSP72 expression was restored only in the group treated with heat within 3 days. The authors suggested that HSP72 may play an important role on muscle remodeling process after skeletal muscle injury.

Heat shock proteins

Heat shock proteins (HSPs) are molecular chaperons that are involved in stress management and intracellular protein homeostasis. For instance, HSPs help proteins achieve a normal three-dimensional framework, prevent accidental, premature, or incorrect folding of polypeptide chains, promote the breakdown of damaged proteins and cooperate with immune cells (30). The expression of HSPs usually increase following heat or mechanical stress on muscle tissue (52). Based on their function and molecular weight, heat shock proteins are classified into several sub-units, i.e. families.

Ubiquitin is a very small HSP (~8kDa) and is known for facilitating degeneration and scavenging necrotic cellular components and denatured proteins following serious injuries. Thompson and Northampton reported that increased free and conjugated ubiquitin levels were observed in biceps muscle 48 hours after 65 eccentric contractions of elbow flexor and highlighted its role in myofibrillar proteolytic pathway and remodeling process in skeletal muscle (87).

Jakob and co-workers determined the roles of small heat shock proteins such as αB-crystallin and HSP27 in response to thermal stress (37). They revealed that HSP27 tends to co-localize with αB-crystallin (~22kDa) in cytoskeleton machinery and both have important roles in preventing irreversible aggregation of apoptotic proteins and facilitating refolding upon the withdrawal of stress.

Morton and colleagues reported significant increases in HSP60 and HSP70 levels and no changes in the level of HSP27 and αB-crystallin in the vastus lateralis muscle 48 hours after non-damaging aerobic exercise (56). They proposed that augmented expression of HSP60 is associated with mitochondrial adaptations following aerobic exercise. In a follow-up study, Morton and colleagues compared resting HSP60 levels between aerobically trained and untrained people (57).

They reported increased HSP60 content in the trained group suggesting again the important role of HSP60 in mitochondrial biogenesis.

HSP70 is a highly conserved protein consisting of several isoforms such as HSC70 and HSP72. The HSP70 family members are known to exert multiple roles in maintaining cellular integrity. For instance, these proteins are involved in transcription factor stabilization and cytoprotection following acute (22) or chronic (47) exercise. Diminished capacity to generate HSP70 with aging seems to be related to skeletal muscle atrophy, loss of force production, and increased risk of muscle damage following contractions (7). Kojima and colleagues investigated the effects of heat stress on muscle regeneration in an animal model of muscle injury caused by cardiotoxin injection on the tibialis anterior muscle (41). They hypothesized that HSPs play key roles in the proliferation and differentiation of muscle stem cells. The level of HSP72 initially decreased following muscle injury and returned to the baseline level within one day in the group treated with heat. Further, Pax7-positive satellite cells and muscle protein content were significantly increased only in the group treated with heat therapy. Although there was no direct relationship between HSP72 level and satellite cell activity, these data showed the beneficial effects of both pre- and post-heat stress on muscle regeneration. Liu and co-workers examined the impact of training intensity on HSP70 expression in human skeletal muscle (46). They separated well-trained rowers into two subgroups and each group underwent two different training phases, which consisted of regimens of different intensity, but matched exercise volume. The expression of HSP70 significantly increased only after the high intensity training phase suggesting that intense exercise bouts may cause muscular damage and the augmented expression of HSP70 may reflect its role in the repair process. Expression of HSP90 families also increases with external stress such as hyperthermia (21) and they are better known to regulate substrate proteins such protein kinases related to angiogenesis.

Effects of heat therapy on regulation of protein synthesis and degradation

Mammalian target of rapamycin (mTOR) is a protein kinase that regulate cell growth and proliferation and influences muscle protein synthesis. For example, the activity of mTOR-related kinases such as ribosomal protein S6 kinase increases following resistance exercise (6) but decreases with detraining (44). Many studies have shown the positive effects of heat therapy on protein turnover, as revealed by an increase in protein synthesis and decrease in protein breakdown.

Yoshihara and co-workers exposed animals to one of five different heat stress regimens (37°C, 38°C, 38°C, 40°C or 41°C), or a control condition without heat stress (97). In both the plantaris and soleus muscles, intense heat stress (40°C and 41°C) upregulated mTOR signaling. Kakigi and co-workers reported that acute exposure to microwave diathermy for 20-min prior to knee extension contractions promoted phosphorylation of Akt, mTOR, and 4E-BP1 following the exercise bout in humans (39). In agreement with these findings, Goto and colleagues reported increased thigh muscle mass and maximum isometric strength following 10 weeks of local heat application (24).

A few studies also indicate that heat application decreases muscle atrophy signaling. Tsuchida and co-workers incubated C2C12 myotubes at 37 or 41°C for 60 min before treating the cells with dexamethasone, a drug that promotes a decrease in myofibrillar protein content and myotube diameter (91). They reported that heat treatment blocked dexamethasone-induced increases in the mRNA expression of MuRF1 and phosphorylation of FoxO1 and FoxO3. A single session of heat treatment at 42°C for 60 min increased rat soleus muscle mass 7 days after the treatment (64). Results of immunoblot analysis showed decreased content of phosphorylated NF-kB and TNFa, both of which are known to negatively regulate skeletal muscle mass by inhibiting myogenic precursor cell differentiation. A recent human study showed that daily heat treatment with pulsed shortwave diathermy attenuates thigh muscle atrophy following 10 days of leg immobilization (26).

Other studies revealed the effects of acute and repeated pre-conditioning heat treatment on muscle protein turnover. Ichinoseki-Sekine and colleagues sought to determine whether whole body heat stress (HS, at 40~41°C for 60 min) performed 24 hours before mechanical ventilation (MV, 12 hours) could protect against diaphragm muscle atrophy (35). They observed significantly decreased actin components in MV group (without HS) along with decreased HSP72 expression and increased oxidative stress and caspase 3 activity compared with the MV+HS group. These findings indicated that pre-conditioning heat stress protects against redox-dependent disuse muscle atrophy. In a follow-up study, the same group tested effects of repeated bouts of HS (1, 3, and 5d before MV) on controlled mechanical ventilation (CMV) induced diaphragm muscle atrophy. The results were in accordance with their previous study (96). Collectively, these studies demonstrated that the protective effects of acute or repeated heat application on muscle protein turnover.

Effects of heat therapy on angiogenesis

The potential effects of heat treatment on angiogenesis have recently received attention. Accumulating evidence from several studies implicates HSPs as important mediators of heat-induced angiogenic signaling. HSP 70 and 90 members are needed to mediate new vessel growth in skeletal muscle. Shiota and coworkers reported that pharmacological inhibition of HSP70s hinders endothelial cell migration and tube formation *in vitro* and blunted new capillary growth in a mouse model of peripheral arterial deficiency (81). Sun and Liao described the central role of HSP90 in regulating key angiogenic signaling derived from Akt and eNOS phosphorylation. Inhibition of HSP90 expression blocked by 17-AAG led to reduced nitric oxide (NO) production followed by impaired NO-dependent angiogenic pathway in cultured human umbilical vein endothelial cells (HUVEC) (83). VEGF is a critical mediator of angiogenesis in skeletal muscle. Following muscle overload, the expression of VEGF increases in association with endothelial cell proliferation and with enhanced expression of matrix metalloproteinase 2 (MMP2) and membrane type 1-matrix metalloproteinase (MT1-MMP). Ihori and colleagues tested whether repeated Waon therapy (far-infrared dry sauna at 39°C for 15 min followed by 34°C for 20 min once daily for 4 weeks) could affect the pathological manifestation of hypertension in the heart in rats. Repeated Waon therapy reduced left ventricular hypertrophy, preserved systolic function of left ventricle, and limited myocardial fibrosis. The positive effects were associated with increased myocardial levels of VEGF and HSP90, suggesting a positive correlation between angiogenic factors and cardiac remodeling following repeated heat treatment.

Effects of heat therapy on mitochondrial adaptation

Heat therapy has also have been reported to enhance mitochondrial function. Mild heat stress (at 40°C for 60 min) applied to C2C12 myotubes increased peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) and transcription factors involved in mitochondrial biogenesis through the AMP-activated protein kinase (AMPK) – sirtuin 1 (SIRT1) – PGC-1α signaling pathway (45). Tamura and colleagues reported that repeated exposure to whole body heat stress (40°C for 30min/day, 5days/week, 3 weeks) increased key mitochondrial enzyme (citrate synthase) activities and respiratory chain proteins in mouse skeletal muscles (86). They also reported the beneficial effects of whole body heat stress on mitochondrial adaptation when combined with endurance training (treadmill running at 25 m/min, 30min/day, 5days/week,

3 weeks). Recently, Hafen and colleagues confirmed that repeated exposure to mild heat stress via shortwave diathermy (2 hours/day for 6 consecutive days) elicited mitochondrial adaptations such as increased PGC-1α and respiratory protein complexes in human skeletal muscle (27).

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CHAPTER 2. HEAT THERAPY PROMOTES THE EXPRESSION OF ANGIOGENIC REGULATORS IN HUMAN SKELETAL MUSCLE

Alisha M. Kuhlenhoelter[1]*, Kyoungrae Kim[1]*, Dustin Neff[1], Yaohui Nie[1, 2], A. Nicole Blaize[1], Brett J. Wong[3], Shihuan Kuang[2], Julianne Stout[4], Qifan Song[5], Timothy P. Gavin[1], Bruno T. Roseguini[1]

[1]Department of Health and Kinesiology, Purdue University, West Lafayette, IN, USA
[2]Department of Animal Sciences, Purdue University, West Lafayette, IN, USA
[3]Department of Kinesiology and Health, Georgia State University, Atlanta, GA, USA
[4]Indiana University School of Medicine – Lafayette, West Lafayette, IN, USA
[5]Department of Statistics, Purdue University, West Lafayette, IN, USA

* Alisha M. Kuhlenhoelter and Kyoungrae Kim have contributed equally to this work

Running Title: Effects of heat therapy on angiogenic factors

Correspondence:
Bruno T. Roseguini, Ph.D.
Department of Health and Kinesiology, Purdue University
800 W. Stadium Avenue, West Lafayette, IN, 47907
E-mail: brosegui@purdue.edu
Phone: 765-496-2612; Fax: 765-496-1239

Abstract

Heat therapy has been shown to promote capillary growth in skeletal muscle and in the heart in several animal models, but the effects of this therapy on angiogenic signaling in humans in unknown. We evaluated the acute effect of lower body heating (LBH) and unilateral thigh heating (TH) on the expression of angiogenic regulators and heat shock proteins (HSPs) in healthy young individuals. Exposure to LBH (n=18) increased core temperature (T_c) from 36.9±0.1 to 37.4±0.1ºC ($p<0.01$) and average leg skin temperature (T_{leg}) from 33.1±0.1 to 39.6±0.1ºC ($p<0.01$), but did not alter the levels of circulating angiogenic cytokines and bone marrow-derived pro-angiogenic cells ($CD34^+CD133^+$). In skeletal muscle, the change in mRNA expression from baseline of vascular endothelial growth factor (VEGF), angiopoietin 2 (ANGPT2), chemokines CCL2 and CX3CL1, platelet factor-4 (PF4) and several members of the HSP family was higher 30 min after the intervention in the individuals exposed to LBH (n=11) as compared to the control group (n=12). LBH also reduced the expression of transcription factor FOXO1 ($p=0.03$). Exposure to TH (n=14) increased T_{leg} from 32.8±0.2 to 40.3±0.1ºC ($p<0.05$) but T_c remained unaltered (36.8±0.09ºC at baseline and 36.9±0.07ºC at 90 min). This protocol upregulated the expression of VEGF, ANGPT1, ANGPT2, CCL2 and HSPs in skeletal muscle but did not affect the levels of CX3CL1, FOXO-1 and PF4. These findings suggest that both LBH and TH increase the expression of factors associated with capillary growth in human skeletal muscle.

Key words: angiogenesis, skeletal muscle, heat therapy, blood flow

Introduction

Skeletal muscle capillary rarefaction is a common feature and a significant contributor to exercise intolerance in several chronic disease states, including peripheral artery disease (PAD) (62), chronic heart failure (CHF) (14) and chronic obstructive pulmonary disease (COPD) (65). Emerging evidence also indicates that a progressive reduction in skeletal muscle microvessel density plays an important role in the development and progression of metabolic syndrome (17). Promoting capillary growth is therefore a major therapeutic goal to restore skeletal muscle function and exercise capacity in these populations. Surprisingly, few therapeutic strategies are known to effectively activate angiogenic signaling and increase skeletal muscle capillarization in humans. Exercise training is undoubtedly one of the most potent angiogenic therapies (18), but few patients engage and adhere to structured exercise programs (47, 61). Further, this option is challenging, or not amenable, for patients with severe disease and restricted locomotion. Gene and cell therapy are promising alternatives, but the results of most therapeutic angiogenesis trials to date have been largely disappointing (2). An urgent need remains for novel, more accessible strategies that stimulate angiogenesis in human skeletal muscle.

Heat therapy is a promising strategy that has recently been successfully used to treat patients with CHF (31, 49), severe COPD (76) and PAD (66, 74). Mounting evidence indicates that repeated exposure to whole-body heat stress reduces the clinical symptoms and improves exercise tolerance in patients with these conditions (31, 49, 66, 76). The exact mechanisms underlying these documented clinical benefits are unclear, but several pieces of evidence indicate that heat therapy might work, in part, by promoting angiogenesis. First, whole-body heat stress induced by far-infrared dry sauna or a heating blanket promotes the expression of the pro-angiogenic mediator vascular endothelial growth factor (VEGF) and increases capillary density in the myocardium in healthy (21) and hypertensive (28) rats as well as in a model of myocardial infarction (69). Second, repeated treatment with far infrared dry sauna increases skeletal muscle capillary density in a mouse model of PAD (1, 40) and diabetes (26) and in rats treated with glucocorticoids (43). This angiogenic response in the heart and in skeletal muscle is closely coupled with increased expression of heat shock proteins (HSPs), molecular chaperones that are known to modulate the angiogenic process (40, 67, 71). Third, endothelial cells exposed to mild heat stress have an enhanced capacity for vascular tube formation, which is indicative of angiogenesis activity (60). Fourth, sauna therapy induces an increase in the number of circulating

$CD34^+$ progenitor cells (49, 66), which promote vascular growth and repair (36). Despite this compelling evidence derived primarily from cell culture and animal studies, the impact of systemic and local heat stress on angiogenic signaling in humans remains to be determined.

Skeletal muscle angiogenesis stems from the cumulative effects of transient changes in the abundance of pro-angiogenic mediators coupled with the inhibition of angiostatic factors (34, 51). Defining the time course and magnitude of changes in the transcriptional levels of these factors in response to a single stimulus is therefore critical to understand how the remodeling process is initiated and coordinated (15, 57). The goal of the present study was to determine the effects of a single 90-min session of heat therapy on systemic and skeletal muscle levels of key angiogenic regulators in humans. Water-circulating trousers were used to create two distinct experimental paradigms: 1) lower body heating (LBH), which induced an increase in body core temperature and significant changes in systemic hemodynamics, and 2) unilateral thigh heating (TH), where the heating stimulus was confined to one thigh and body core temperature remained unaltered. These distinct experimental approaches allowed us to examine the potential contribution of systemic responses to heat stress to local changes in the expression of angiogenic genes in skeletal muscle. Systemic heat stress has been shown to induce physiological responses that can impact skeletal muscle angiogenesis, including activation of the sympathetic nervous system (7) and increased levels of bone marrow-derived pro-angiogenic cells ($CD34^+CD133^+$) (49, 66). Therefore, we hypothesized that LBH would increase the circulating levels of pro-angiogenic cytokines and cells and promote an increase in the mRNA level of angiogenic regulators and HSPs in skeletal muscle. Since a systemic angiogenic response would not occur during TH, we hypothesized the changes in the expression of pro-angiogenic factors in skeletal muscle following this protocol would be smaller when compared to LBH.

Methods

Participants

Fifty-five young, normally active individuals (Table 1) were recruited to participate in three separate studies (Protocol 1, n=18; Protocol 2, n=23; and Protocol 3, n=14). Participants were asked to fill out a medical history questionnaire. Individuals were excluded if they were obese (BMI>30 kg/m^2), used tobacco products, were diabetic, were taking any medication other than birth control, participated in any kind of supervised physical activity or exercised for more than 3

days/week. Female participants were tested during the early follicular phase of their menstrual cycle (days 1-7) or during the placebo phase if they were taking oral contraceptives. The Institutional Review Board at Purdue University approved all experimental procedures and verbal and written consent were obtained from all participants.

Instrumentation and experimental protocols

Three separate protocols were conducted to determine the angiogenic responses to LBH (protocols 1 and 2) and TH (protocol 3). The experimental design with time points for blood draws and muscle biopsies for each protocol is depicted on Figure 1. All experimental sessions were completed in the morning in a temperature-controlled room (average temperature of 24.2±0.2ºC). Participants were asked to avoid caffeine and alcoholic beverages for 12 hrs and intense exercise for at least 24 hrs prior to the experimental sessions. Participants were also asked to abstain from food for at least one hour prior to each laboratory visit.

The purpose of *protocol 1* was to determine the effect of LBH on the circulating levels of angiogenic, inflammatory, and vasoactive mediators as well as the number of circulating pro-angiogenic cells. Participants completed two experimental sessions, at least 72 hours apart, in a randomized, cross-over design. Subjects were given a wireless telemetry pill (HQ Inc, Palmetto, FL) for core temperature monitoring during the experiments and were instructed to ingest it the night before the experiment (~7-9 hrs before the experimental sessions) (82). Upon arrival at the laboratory, subject body weight and height were recorded and four thermocouples (MLT422, ADInstruments, Colorado Springs, CO) were taped to the calf and thigh for measurement of mean leg skin temperature. The medial aspect of the calf on both legs was gently shaved and laser Doppler flow probes (VP12, Moor Instruments, Axminster, UK) placed inside local heaters (VHP1, Moor Instruments, Axminster, UK) were attached to the skin for the measurement of skin red blood cell flux (moorVMS- LDF2, Moor Instruments, Axminster, UK). Heart rate (HR) was monitored via a 3-lead electrocardiogram (FE132, Bio Amp, ADInstruments, Colorado Springs, CO). Systolic and diastolic blood pressures (BP) were measured from the left arm using an automated device (Tango+, Suntech Medical, Morrisville, NC). An intravenous catheter was placed in an antecubital vein of the right arm for blood sampling. Participants were asked to put on water-circulating trousers on top of shorts or underwear (Med-Eng, Ottawa, Canada). This garment is made of a tight-fitting elastic fabric, with an extensive network of medical grade

polyvinyl chloride tubing sewn onto the fabric and was designed to cover the calves, thighs and buttocks (Figure 1, left panel). In addition, to minimize heat loss during the interventions, participants also wore polyvinyl chloride pants and had their legs covered with a thermal foil blanket. After instrumentation, participants rested quietly for 30 min in the supine position and a baseline blood sample was taken. Next, the water-circulating garment was connected to a heated bath circulator (Sahara S21, Thermo Scientific). In the LBH session, water at 48ºC was perfused through the garment for 90 min with a goal to increase leg skin temperature to ~39.5-40ºC (24). In the control intervention, water at 33ºC was circulated through the garment to maintain leg skin temperature at baseline levels. At the end of the intervention period, the garment was disconnected from the water circulator and the participants remained supine for another 2 hrs. Blood samples were taken 30 and 120 min following the completion of the trials (Figure 1, left panel). In the last 20 min of the protocol, the temperature of the local skin heaters placed on the calf were increased to 43ºC at a rate of 0.1ºC/s to promote maximal increases in skin blood flow and allow for the calculation of maximal cutaneous vascular conductance (CVCmax) (11). Leg skin temperature, cutaneous red blood cell flux, and HR were recorded continuously, while systolic and diastolic BP and core body temperature were recorded every 5 min for the entire duration of the protocol.

In *protocol 2*, enrolled participants were randomly allocated to either a group exposed to LBH (n=11) or to a control group (n=12). The purpose of this protocol was to determine the effect of LBH on the mRNA expression of angiogenic mediators in skeletal muscle. Participants were instrumented as described above in *protocol 1* (Figure 1, left panel). After 30 min of rest in the supine position, the thighs were exposed and a biopsy was taken from the vastus lateralis muscle of a randomly selected thigh using a 5-mm Bergstrom biopsy needle (Pelomi Medical, Albruslund, Denmark) as described previously (19, 20, 64). Next, the water-circulating garment was perfused with either 48ºC water (LBH group) or 33ºC water (control group) for 90 min. Two additional muscle biopsies were taken 30 and 120 min after the end of the intervention (Figure 1, left panel). The second biopsy was taken from the opposite leg used for the first biopsy and the third biopsy was taken from a site at least ~3 cm away from the first biopsy site.

In *protocol 3*, a custom-made garment was used to heat one thigh while the opposite thigh served as a control (Figure 1, right panel). The goal of this experiment was to investigate the effects of local heating, which does not evoke systemic responses such as changes in core body temperature, on the mRNA expression of angiogenic mediators in the vastus lateralis muscle.

Participants were asked to complete two experimental sessions. On the first session, two skin thermocouples were taped to each thigh for measurement of skin temperature and participants were asked to put on the custom water-circulating garment and polyvinyl chloride pants and had their legs covered with a thermal foil blanket. A baseline muscle biopsy was taken after 30 min of rest in the supine position from one randomly selected thigh. Next, the garment was connected to the water circulators. In the thigh assigned to receive the heat treatment, hot water (48-52ºC) was circulated through the garment for 90 min to increase skin temperature to ~39-40ºC as in protocols 1 and 2. In the opposite thigh, skin temperature was clamped at 33ºC for the entire duration of the protocol. Thirty minutes after the end of the treatment, one muscle biopsy was taken from both the control and heated thighs as described above (Figure 1, right panel). The biopsies were only taken at 30 min after the intervention in this protocol because most of the changes in mRNA levels occurred at this time point in protocol 2. The second session was conducted at least one week after session 1. The purpose of this session was to characterize the physiological responses to TH. Subjects were instructed to ingest the wireless telemetry pill (HQ Inc, Palmetto, FL) for core temperature monitoring as described for protocol 1. Upon arrival, subject weight and height were recorded and skin thermocouples were taped to the thigh for the measurement of skin temperature. Participants were then fitted with the custom-garment and polyvinyl chloride pants as detailed above. A blood pressure cuff was wrapped around the left arm for blood pressure monitoring during the protocol using an automated device (Tango+, Suntech Medical, Morrisville, NC). After instrumentation, subjects were allowed to rest quietly for 30 min in the supine position. Next, the groin area was exposed and the common femoral artery was imaged on both legs using a 10-MHz multifrequency linear probe attached to a high-resolution ultrasound machine (T3000; Terason, Burlington, MA). Diameter and velocity signals were acquired simultaneously in duplex mode and corrected with an insonation angle of 60º. Images were recorded for 1 min in each leg using screen capture software (Camtasia Studio, TechSmith). The garment was then connected to the water circulators for application of local heating and control treatments as described above. Diameter and blood flow recordings were made every 30 min during the intervention and once 30 min after the end of the heating protocol. Blood pressure and core body temperature were measured every 5 min, while skin temperature was recorded continuously for the entire duration of the protocol.

Measurements

Circulating factors:

Venous blood samples were collected in serum-separating tubes (BD Vacutainer SST plus), allowed to clot for 30 min at room temperature, centrifuged for 15 min at 1200 rpms, aliquoted and stored at -80ºC until analysis. A custom Milliplex® assay kit (EMD Millipore Corp, Billerica, MA) was used to determine the concentrations of several angiogenic and inflammatory mediators, including: granulocyte colony stimulating factor (G-CSF), granulocyte macrophage stimulating factor (GM-CSF), interleukin 6 (IL-6), interleukin 8 (IL-8), chemokine (C-C motif) ligand 2 (CCL2), tumor necrosis factor alpha (TNF-α), vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2) and chemokine (C-X3-C motif) ligand 1 (CX3CL1). The assay was performed following the manufacturer's protocol using the Bio-Plex 200 System (Bio-Rad, CA) at the Bio-Plex Core Facility of Indiana University. The serum concentration of endothelin-1 was determined using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) according to manufacturer's instructions.

Circulating pro-angiogenic cells

A multiparametric flow cytometry method (45, 46) was used to identify circulating $CD34^{+}CD133^{+}$ cells. Briefly, venous blood samples were collected in a cell preparation tube with sodium heparin (BD Vacutainer CPT™ tubes) and taken to the Flow Cytometry and Cell Separation Facility (Bindley Bioscience Center, Purdue University). Samples were centrifuged within two hours of collection for 15 min at 1500 rcfs as suggested by the manufacturer. Mononuclear cells were isolated, washed in phosphate-buffered saline (PBS) with 0.5% bovine serum albumin (BSA) and stained with antibodies against cell surface antigens, including: anti-CD34-PE (BioLegend, # 343506), anti-CD31-FITC (BioLegend, # 303104), anti-CD45-BV510 (BioLegend, # 304014) and anti-CD133-APC (Miltenyi, #130-090-826). In addition, in order to exclude dead/apoptotic cells, red blood cells and monocytes, mononuclear cells were co-stained with Live/Dead stain-violet (Life Technologies, #L34955), anti-CD235a-Pacific Blue (BioLegend, #306612), and anti-CD14-BUV395 (BD Biosciences, #563561), respectively. The cells were initially stained with Live/Dead stain for 30 min at 4ºC, washed once with PBS/BSA, incubated with antibodies for 20 min at 4ºC, washed twice in PBS/BSA and were analyzed using a BD

FACSAria III Cell Sorter. At least 1,000,000 events were collected for each sample. The data was analyzed using Flowjo v9.4.3 (Treestar).

Skeletal muscle gene expression

Biopsy samples were initially placed in a microcentrifuge tube containing 1 mL of RNAlater® (Ambion, Carlsbad, CA). The sample was kept at 4ºC overnight and then stored at -80ºC until analysis. Approximately 30 mg of tissue was used for RNA extraction using the TRIzol Reagent (Invitrogen, Life Technologies, Carlsbad, CA) according to manufacturer's instructions. The quality and concentration of total RNA were measured by a spectrophotometer (Nanodrop 3000, ThermoFisher), as described previously (84). cDNA was prepared using the RT^2 First Strand Kit following the manufacturer's instructions (Qiagen, Valencia, CA). In *protocol 2*, the expression of 27 genes related to angiogenesis and inflammation as well as members of the HSP family were determined using a custom RT^2 Profiler PCR array kit (Qiagen, Valencia, CA) and the Roche LighCycler 480 PCR System (Roche Diagnostostics, Indianapolis, IN). The three samples from each subject (baseline, 30 min and 120 min post intervention) were loaded in the same array plate. The list of genes analyzed is shown on Table 2 and included the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as well as a reverse-transcription control, a positive PCR control and a genomic DNA control. Data was analyzed using the GeneGlove Data Analyses Center (Qiagen, Valencia, CA). GAPDH was used to normalize cycle threshold (Ct) values. The comparative Ct method was used to calculate the changes in gene expression of each target mRNA relative to the baseline sample (33). A similar strategy was used to analyze the samples obtained in *Protocol 3*, with the exception that the custom array had only 13 select genes (Table 3).

Data and statistical analysis:

Leg skin temperature, cutaneous red blood cell flux and HR were recorded at 40 Hz using a data acquisition system (Powerlab and LabChart, ADInstruments, Colorado Springs, CO) and the last 2 min of every 5-min bins were averaged for the entire protocol. Systolic and diastolic BP were recorded every 5 min and mean arterial pressure was calculated as diastolic pressure plus one-third pulse pressure. Cutaneous vascular conductance (CVC) was calculated as red blood cell

flux divided by mean arterial pressure and expressed as a percentage of CVCmax, as determined by the maximal value obtained during local heating at 43ºC for 20 min.

Analysis of diameter and Doppler flow profile in the femoral artery in protocol 3 was performed using computerized edge-detection and wall-tracking software (FMD Studio, Quipu, Pisa, Italy). The Doppler flow analysis computed time average anterograde (V_{ant}) and retrograde (V_{ret}) values of velocity (V), which were used for the calculation of positive and negative blood flows (BF) using the following equation: $BF=(V \times 3.14 \times d^2)/400$. Mean BF was computed as the algebraic sum of anterograde and retrograde BF. Anterograde and retrograde wall shear rates (SR) were calculated using the following equation: $SR= 4 \times V/d$, where d is the vessel diameter. The oscillatory shear index (OSI) was calculated as: $OSI=(SR_{ret})/(SR_{ant} + SR_{ret})$.

All statistical analyses were conducted using SAS (Version 9.4; SAS Institute) with results expressed as means ± SE. A two-way mixed effect (with random individual effect and repeated measurements) ANOVA was used to compare the physiological responses and the levels of circulating factors and progenitor cells between trials in protocol 1, followed by Bonferroni post hoc comparisons when appropriate. In protocol 2, subject characteristics were compared between groups using unpaired *t*-tests. Due to non-normal distribution, gene expression responses at each time point (30 min and 120 min) were compared between groups using the Wilcoxon Rank-Sum Test. In protocol 3, a one-way repeated-measures ANOVA model was used to identify significant changes from baseline in heart rate, systolic and diastolic BP and skin and core body temperature. A two-way mixed effect ANOVA was employed to compare the blood flow parameters between the leg exposed to TH and the control leg. Post-hoc analysis (Bonferroni) was performed when appropriate. As in protocol 2, gene expression responses had a non-normal distribution in protocol 3 and differences between thighs were compared using the paired Wilcoxon rank test. To allow for a direct comparison between gene expression responses at 30 min post-intervention between protocols 2 and 3, each variable was subjected to logarithm transformation. A t-test was then used to compare the changes in the expression of each gene between the two distinct interventions. For all analyses, $P \leq 0.05$ was considered statistically significant.

Results

Protocol 1

Physiological responses to LBH

Figure 2 depicts the changes in skin and core temperatures, HR, systolic and diastolic blood pressure, and CVC before, during and after exposure to LBH or a control intervention. There were no baseline differences between trials for any of the physiological variables. As expected, exposure to LBH for 90 min induced marked increases from baseline in leg skin temperature (from 33.1±0.1 to 39.6±0.1ºC, $p<0.01$), CVC (from 22.9±3.0 to 56±3.0%max, $p<0.01$) and HR (from 67±2 to 87±2 bpm, $p<0.01$) as well as a modest increase in core temperature (from 36.9±0.1 to 37.4±0.1ºC, $p<0.01$). Systolic blood pressure was not affected by LBH, but diastolic blood pressure was significantly lower during the last 30 min of heating and early in the recovery period after LBH when compared to the control trial ($p<0.05$, Figure 2, Panel F).

Effect of LBH on circulating cytokines and bone marrow-derived pro-angiogenic cells

The serum concentrations of angiogenic and inflammatory mediators and the percentage of pro-angiogenic cells ($CD34^+CD133^+$) measured prior to and 30 and 120 min following exposure to LBH or the control intervention are shown on Table 4. The concentration of three (IL-6, CX3CL1 and GM-CSF) out of the 9 factors measured was at or below detection levels and therefore the data were excluded. There were no differences between trials for any of the measured growth factors, cytokines or the percentage of $CD34^+CD133^+$ cells. The concentration of the potent vasoconstrictor ET-1 was significantly lower at 30 min following the intervention in the LBH trial compared to the control condition ($p<0.01$) (Table 4).

Protocol 2

Effect of LBH on the mRNA levels of angiogenic and inflammatory mediators in skeletal muscle

Changes in gene expression from baseline for select angiogenic and inflammatory genes are shown on Figure 3 and Table 5. The expression of 3 genes (IL8, TNF and MMP9) was near or below detection limits (i.e. Ct values ≥ 35 or not detectable) and the data were excluded. The mRNA expression of pro-angiogenic factors VEGF and ANGPT2 were increased at 30 min in the group exposed to LBH when compared to the control group ($p<0.05$) (Figure 3). ANGPT1 expression was not significantly different between groups. The mRNA expression of chemokine

CCL2 was higher in the LBH group at 30 min following the intervention (p=0.02), while the levels of chemokine CX3CL1 were higher at both 30 and 120 min following LBH when compared to the control group (p<0.05). The expression of the angiostatic transcription factor FOXO1 was lower in the LBH group at 120 min following the end of intervention period (p=0.03). Conversely, the mRNA expression of the angiostatic factor PF4 was increased at 30 min in the LBH group relative to the control group (p=0.03). The fold changes in mRNA expression of eNOS, PPARGC1A, MMP2, TIMP1, TEK, THBS1, CXCL12, NCL, FOXO3 and IL6 were not different between groups (Table 5).

Effect of LBH on the mRNA levels of heat shock proteins

The relative mRNA expression of members of several HSP families is shown on Figure 4. In agreement with what was observed for the angiogenic/inflammatory genes, increased expression of these factors in the LBH group was particularly evident at 30 min following the intervention. The mRNA levels of HSP90AA1 and HSP90AB1, members of the HSP90 family, were higher in the LBH group compared to control group at 30 min (p<0.01; figure 4), but there were no differences between groups at 120 min. The expression of HSPA1A, also termed HSP72 and a member of the HSP70 family, was slightly higher in the LBH group at 30 min (Fold change, Control: 0.8±0.04 and LBH: 1.06±0.09) but this difference did not reach statistical significance (p=0.08; table 5). The expression of HSPA1B and HSP70, both members of the HSP70 family, was higher in the group exposed to LBH than in the control group at 30 min (p<0.05; figure 4). Similarly, the mRNA levels of mitochondrial encoded HSPD1, a member of the chaperonin family, was higher at 30 min in the group exposed to heat treatment than in the group exposed to the sham intervention (p=0.018; figure 4).

Protocol 3

Physiological responses to TH

Skin temperature and body core temperature responses to TH are shown on Figure 5. By study design, skin temperature in the thigh exposed to local heating increased to ~40ºC during the treatment, while in the control leg, the temperature was clamped at ~33ºC. Core temperature remained constant throughout the entire protocol. Systolic and diastolic BP and HR were also not significantly altered by TH treatment (data not shown). Compared to the control leg, mean BF and

anterograde SR in the femoral artery increased during TH, while retrograde SR and OSI were markedly reduced during the treatment (Table 6).

Effect of TH on the mRNA levels of angiogenic mediators in skeletal muscle

Changes in gene expression from baseline for select angiogenic genes following local heat treatment are shown on Figure 6. In congruence with the findings from protocol 2, TH led to increased mRNA expression of VEGF, ANGPT2 and CCL2 relative to the control thigh ($p<005$). In contrast to LBH, localized heating increased the levels of ANGPT1 ($p<0.05$) but did not affect the expression of CX3CL1 ($p=0.16$), FOXO1 ($p=0.85$) and PF4 ($p=0.37$).

Effect of TH on the mRNA levels of heat shock proteins

In close agreement with the changes induced by LBH, TH increased the expression of members of the HSP90 family (HSP90AA1 and HSP90AB), HSP70 family (HSPA1B and HSPA8) and the chaperonin family (HSPD1) (Figure 7).

Comparison of skeletal muscle gene expression responses between LBH and TH

The comparison between the LBH and TH protocols for the changes in the expression of select angiogenic factors and heat shock proteins 30 min after the interventions is shown on Table 7. Contrary to our initial hypothesis, the magnitude of change in the expression of these factors was not different between the two interventions.

Discussion

In the present study, we employed two experimental paradigms to determine the effect of a single session of heat therapy on the expression of angiogenic mediators and HSPs in humans. Mild systemic heat stress was induced by circulating 48ºC water through a garment designed to cover the legs, thighs and buttocks. This strategy increased core body temperature by ~0.6ºC and reduced diastolic BP and the serum concentration of the potent vasoconstrictor ET-1. Despite inducing these systemic effects, LBH did not affect the circulating levels of several cytokines or bone marrow-derived pro-angiogenic cells ($CD34^{+}CD133^{+}$). Conversely, in skeletal muscle, LBH evoked a transient increase in the mRNA expression of several important angiogenic factors, including VEGF and HSPs. To explore the mechanisms underlying this response, we designed a

custom water-circulating garment that allowed for localized heat application to one thigh while the contralateral thigh served as control (Figure 1, right panel). In this model, body core temperature and systemic hemodynamics were not significantly altered, while femoral blood flow in the heated thigh increased markedly compared to the control thigh. In close agreement with the responses induced by LBH, local heating also upregulated the expression of angiogenic factors and HSPs in skeletal muscle. These findings suggest that the acute angiogenic response to LBH stems largely from local mechanisms and is not influenced by the systemic responses induced by this treatment. Overall, this study reveals that heat therapy is a simple, non-invasive strategy to activate local angiogenic signaling and possibly promote vascular growth in skeletal muscle.

Effects of heat therapy on circulating angiogenic factors and pro-angiogenic cells

The reported increase in circulating levels of bone marrow-derived $CD34^+$ cells in patients with PAD (66) and CHF (49) after treatment with sauna therapy prompted us to investigate the acute effects of LBH on the numbers of these cells as well as on the concentrations of angiogenic cytokines that participate in the recruitment and mobilization of progenitor cells. The angiogenic potential of $CD34^+$ cells has been well documented in numerous clinical and pre-clinical studies (36) and appears to be related to the ability of these cells to produce and secrete angiogenic cytokines (37). Using a multiparametric flow cytometry protocol, we identified and quantified the number of cells that co-express CD34 and the primitive stem cell marker CD133 (83). Contrary to our hypothesis and the aforementioned sauna studies (49, 66), a single session of LBH did not affect the levels of circulating cytokines and pro-angiogenic cells (Table 2). Combined, these findings indicate that acute LBH does not evoke a systemic angiogenic response in healthy young participants. Since sauna therapy typically induces larger changes in core temperature (~1-1.2ºC) (73) than what we observed in the present study (~0.6ºC), one potential explanation for the lack of effect of LBH on the systemic levels of these proangiogenic mediators is that the heat stress level was not sufficient to trigger the production and/or release of cytokines and promote the recruitment of progenitor cells. Another possibility is that 2 hours was not long enough after LBH to induce changes in the levels of these circulating pro-angiogenic factors and cells. In addition, it is conceivable that multiple or longer sessions of LBH are necessary to effectively activate these angiogenic mediators.

Skeletal muscle angiogenic signaling

Several animal studies demonstrated unequivocally that heat therapy promotes angiogenesis in the heart (21, 28, 69) and in skeletal muscle (1, 40, 43), but the molecular mechanisms underlying this adaptation remain poorly defined. One attractive candidate is VEGF, a potent angiogenic inducer that acts by promoting proliferation and enhancing migration and invasion of endothelial cells (5). Increased capillarization in the myocardium following whole-body heat stress in rats is indeed associated with a marked up-regulation of VEGF expression (21, 28, 69). In the present study, an increase in VEGF mRNA expression in skeletal muscle was observed following both LBH and TH, demonstrating that this central pro-angiogenic factor might also mediate the angiogenic response to heat therapy in skeletal muscle. VEGF regulates basal and exercise training induced increases in skeletal muscle capillarization (80) and muscle-specific deletion of this molecule leads to capillary rarefaction (3, 52) and nearly abolishes exercise-induced capillary growth (53).

In addition to VEGF, heat therapy activates other key players involved in the angiogenic cascade. ANGPT1 and ANGPT2 are important pro-angiogenic factors that act as ligands for Tie-2 receptors in endothelial cells (16). Although the importance of angiopoietin signaling in the vasculature is well defined, the cellular origins and functional roles of these two factors in skeletal muscle have only recently received attention. ANGPT1 is the principal angiopoietin produced by skeletal myoblasts and myotubes and is an important regulator of myogenesis and angiogenesis (38, 42). Conversely, ANGPT2 has no effect on myoblast proliferation and migration and exerts a relatively weak and context-dependent effect on angiogenesis (41). Mofarrahi and co-workers recently showed that overexpression of ANGPT1 following muscle injury in mice promoted capillary growth and accelerated the recovery of contractile performance (42). In this scenario, it is noteworthy that TH induced the expression of ANGPT1 in skeletal muscle (Figure 6). This novel finding might help explain why repeated heat therapy application facilitates regeneration of injured skeletal muscle (50, 72).

Individuals exposed to LBH had higher mRNA levels of CCL2 and CX3CL1 relative to the control group (Figure 3). CCL2 expression was also higher in the heated thigh compared to the control thigh on protocol 3 (Figure 6). This is an important finding because these myokines/chemokines are involved in the attraction of monocytes/macrophages, which are critical for skeletal muscle remodeling (8). Impaired CCL2 signaling via deletion of its receptor, CCR2,

delays angiogenesis and reduces VEGF levels following skeletal muscle injury in mice (48). Recently, Strömberg and co-workers demonstrated that CX3CL1 stimulation of primary human myoblasts and myotubes promoted marked increases in the expression of proangiogenic factors and chemotactic mediators (70). These observations, coupled with the notion that the expression of both factors increase markedly in skeletal muscle following an acute bout of exercise in humans (6), indicate that these chemoattractants promote a microenvironment that facilitates angiogenesis and muscle repair (70).

The expression levels of the transcription factor FOXO1 were reduced two hours after the intervention in the group treated with LBH. FoxO1 regulates the transcription of several angiostatic factors and acts to restrain angiogenesis in skeletal muscle (63, 68). A reduction in the expression of this factor might therefore allow for the initiation of the angiogenic cascade. Altogether, these findings indicate that both LBH and TH promote the expression of some pivotal factors that mediate capillary growth in skeletal muscle. It is conceivable that these acute responses might translate over time to increased capillary supply in skeletal muscle after repeated treatment. Indeed, evidence derived from exercise training studies indicates that skeletal muscle angiogenesis is preceded by repeated transient mRNA bursts of growth factors and pro-inflammatory mediators (23, 34). Nonetheless, it is important to highlight that the changes in the expression of some factors in the present study were transient and relatively small. Whether protein levels of these pro-angiogenic factors are also altered by acute heat therapy remains to be determined. Additional studies are needed to explore the long-term effects of repeated heat therapy on skeletal muscle capillarization in healthy and diseased populations.

One important observation of the current study was that the expression of some genes were altered in the control group on protocol 2 and in the thigh exposed to the control intervention on protocol 3. This response was particularly evident for CCL2 and CX3CL1, which were consistently downregulated following the control intervention on both protocols (Figures 3 and 6). This response might be potentially explained by circadian oscillations in gene expression (56), changes in metabolic status (79) or forced physical inactivity for a long period of time. In agreement with our findings, prior studies have demonstrated the mRNA expression of CX3CL1 tends to decrease slightly over time in resting/non-exercising skeletal muscle (6, 70). Intriguingly, however, the expression of CCL2 has been reported to increase, albeit not significantly, in resting muscle 2-4 hours following a baseline biopsy (6, 70, 78). These changes in the expression of key

myokines illustrate the critical importance of incorporating a control group in long experiments to account for time-dependent variations in skeletal muscle gene expression in humans.

Heat shock proteins and angiogenesis

Most of the salutary effects of heat stress on skeletal muscle are thought to be mediated, in part, by HSPs (25). This highly conserved family of proteins function as molecular chaperones and is involved in multiple cellular activities, including angiogenesis signaling (58, 67, 71). For instance, members of the HSP70 and HSP90 families have been reported to modulate angiogenesis in skeletal muscle (58, 67). Pharmacological blockade of HSP70s impairs endothelial cell migration and tube formation *in vitro* and abrogates capillary growth in a model of peripheral arterial insufficiency (67). Similarly, administration of HSP90 inhibitor 17-DMAG suppressed the angiogenic response to repeated dry infrared sauna therapy in mice with hindlimb ischemia (40). The HSP90 inhibitor 17-AAG has also been shown to impair migration and formation of capillary-like tubes in cultured human umbilical vein endothelial cells (HUVEC) (71). Given the importance of these factors for angiogenesis, it is surprising that few studies have determined the impact of passive heat stress on the expression of HSPs in skeletal muscle in humans. Morton and co-workers showed that leg immersion in water at 45ºC for 1 hour had no effect on muscle protein content of HSPs in healthy young volunteers (44). Vardiman and co-workers also found that a single session of local leg heating with fluidotherapy did not alter the content of HSP70 and HSP27p in skeletal muscle (77). In contrast, Touchberry and co-workers reported that local heating with diathermy and hot packs increases the skeletal muscle content of HSP70 and HSP27p in female but not male subjects (75). Our study focused solely on the immediate transcriptional responses of several members of distinct HSP families. We report that both LBH and TH promote a rapid and very consistent up-regulation of the mRNA expression of HSPs in human skeletal muscle, including members of the HSP90 and HSP70 families (figures 4 and 7). As these proteins have been shown to modulate angiogenesis in skeletal muscle, it is conceivable that the angiogenic response to heat therapy is regulated and potentially dependent on heat-induced activation of HSPs.

Potential mechanisms

One hypothesis of the present study was that the skeletal muscle angiogenic response to LBH would be greater than that induced by TH, because in addition to activating local pro-

angiogenic signals, systemic heat stress promotes physiological responses that can influence angiogenesis. For example, as discussed above, whole-body heat stress has been shown to trigger the release and recruitment of bone marrow derived pro-angiogenic cells (49, 66). Further, whole-body heat stress evokes a marked increase in sympathoadrenal activity and an associated elevation in circulating levels of catecholamines (27). Catecholamines have been previously shown to contribute to skeletal muscle angiogenesis in a model of peripheral vascular insufficiency (7). Contrary to our initial predictions, exposure to LBH did not alter the levels of cytokines and pro-angiogenic cells and most importantly, the increase in mRNA expression of angiogenic factors in skeletal muscle was remarkably similar between LBH and TH (Table 7). These findings suggest that the angiogenic response to both strategies derive from the activation of local signals capable of initiating the angiogenic cascade. One such mechanism would be the increase in skeletal muscle blood flow and shear-stress that occurs during heat therapy application. Increased shear stress is known to induce the expression of several pro-angiogenic genes and provoke remodeling of the skeletal muscle microvasculature (4). The observed progressive increase in blood flow and shear rates in the femoral artery during TH (Table 6) is consistent with other recent reports (9, 10). Of note, the magnitude of change in leg blood flow is remarkably similar during isolated leg and moderate whole-body heating, which indicates that this hyperemic response is controlled by local mechanisms (9, 10). Until recently, the prevailing view was that these increases in bulk limb blood flow in response to heat stress were solely the result of changes in skin blood flow (see (12) for review). However, following the seminal report of Keller and co-workers (29), numerous studies have provided evidence that skeletal muscle blood flow also increases during local hyperthermia (10, 24, 55). For example, Heinonen and co-workers showed using positron emission tomography that leg heating increases blood flow in the gastrocnemius muscle by ~1 mL/min/100g (24). Although this change is considered small when compared to the hyperemic responses induced by interventions such as exercise (12), it is fair to speculate that sustained increases in local flow during 90 min of heat therapy application and in the recovery period might induce a local change in the expression of angiogenic factors.

On the other hand, emerging evidence indicate that heat stress can promote angiogenesis independent of changes in blood flow. Rattan and co-workers first showed that pre-exposure of HUVEC and human dermal microvascular endothelial cells to mild heat stress improved their ability to form capillary-like tubes (60). More recently, Li and co-workers demonstrated that mild

heat stress increased the expression of VEGF and angiopoietins and enhanced the formation of microvessel-like structures in a co-culture system of outgrowth endothelial cells and osteoblasts (32). These authors speculate that this potent angiogenic response is related to the aforementioned heat-induced expression of HSPs (59), which are known to independently regulate angiogenesis in cultured cells (71) and in animal models (40, 67).

Clinical implications

Heat therapy can be applied using several different modalities, including dry sauna (39), immersion in warm water (54), perfusion of hot water through a tube-lined garment (12) and other methods that are commonly used in rehabilitation settings, such as diathermy (75) and fluidotherapy (77). The use of liquid-circulating garments is attractive because this approach is simple, inexpensive, portable and amenable for home-based application, which makes this option particularly suitable for patients with limited mobility. Although we choose to apply the treatment to the lower limbs, it is important to highlight that these garments can be customized to cover just about every region of the body. For instance, tube-lined suits that cover the whole body allow for a higher rate of heating and more significant changes in body core temperature than observed in the current study (12). This modality has been extensively employed to investigate thermoregulatory responses in older individuals (22) as well as in patients with hypertension (30), type 2 diabetes (81) and chronic heart failure (13). It is conceivable that the higher magnitude of heat stress attained by this method could induce responses that are different than the ones evoked by targeting just the lower limbs.

On the other hand, local heating methods such as the TH protocol used herein afford the possibility of inducing substantial increases in tissue temperature while maintaining body temperature stable. These strategies might be more appealing for patients that do not tolerate or have contra-indications to whole-body heat stress. Regardless of whether heat treatment is applied locally or systemically, the findings from the present study indicate that this technology may serve as a practical approach to stimulate angiogenic signaling and promote vascular growth in skeletal muscle.

Limitations

One potential limitation of our experimental design was that subjects were not allowed to drink water during the experiment and it is possible that sweat-induced dehydration during the LBH protocol might have contributed to the observed changes in skeletal muscle gene expression. However, this confounding effect seems unlikely given that changes in the expression of angiogenic factors and heat shock proteins were remarkably similar between the LBH and TH protocols (Table 7), even though there is negligible dehydration during localized limb heating. In addition, Logan-Sprenger and co-workers recently reported that mild dehydration induced by overnight fluid restriction had no significant impact on the expression of HSP72 in skeletal muscle in humans (35). Taken together, these observations indicate that mild dehydration does not seem to impact the expression of angiogenic factors and heat shock proteins in skeletal muscle. It remains to be determined whether more severe levels of dehydration can affect the expression of these factors in humans.

Perspectives and significance

Our results show that heat therapy, applied either to both legs or locally to one thigh of healthy young individuals, promotes the expression of key angiogenic mediators in skeletal muscle. These findings set the stage for future studies to test the hypothesis that repeated exposure to this therapy can lead to increased capillarization in healthy and diseased skeletal muscle. If this hypothesis is confirmed, heat therapy may become a practical, non-invasive therapeutic tool to reverse the rarefaction of the skeletal muscle capillary network that is commonly observed in individuals with chronic diseases such as COPD, PAD and CHF.

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Conflicts of interest:

None

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Table 2.1: Subject characteristics

	Protocol 1	**Protocol 2**		**Protocol 3**
		Control (33°C)	**LBH (48°C)**	
Number of subjects (male/female)	18 (12/6)	12 (8/4)	11 (6/5)	14 (12/2)
Age (years)	22 ± 1.1	21 ± 0.6	24 ± 1.7	21 ± 0.8
Body weight (kg)	70.3 ± 2.9	72.4 ± 4.6	70.7 ± 4.3	73.4 ± 3.0
Height (cm)	172.2 ± 1.5	171.0 ± 2.5	173.5 ± 4.0	175.0 ± 2.4
BMI (kg/m^2)	23.7 ± 0.9	24.6 ± 1.2	23.3 ± 0.9	23.9 ± 0.8

Values are as mean ± SE. BMI, body mass index.

Table 2.2: List of genes included in the custom PCR array used in protocol 2

Gene symbol	Official full name
VEGFA	Vascular endothelial growth factor A
NOS3	Nitric oxide synthase 3
PPARGC1A	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha
CCL2	Chemokine (C-C motif) ligand 2
MMP9	Matrix metallopeptidase 9
MMP2	Matrix metallopeptidase 2
TIMP1	TIMP metallopeptidase inhibitor 1
ANGPT2	Angiopoietin 2
ANGPT1	Angiopoietin 1
TEK	TEK tyrosine kinase, endothelial
THBS1	Thrombospondin 1
PF4	Platelet factor 4
CXCL12	Chemokine (C-X-C motif) ligand 12
NCL	Nucleolin
FOXO1	Forkhead box O1
FOXO3	Forkhead box O3
IL6	Interleukin 6
IL8	Interleukin 8
TNF	Tumor necrosis factor
CX3CL1	Chemokine (C-X3-C motif) ligand 1
HSP90AA1	Heat shock protein 90kDa alpha class A member 1
HSP90AB1	Heat shock protein 90kDa alpha class B member 1
HSPA1A	Heat shock 70kDa protein 1A
HSPA1B	Heat shock 70kDa protein 1B
HSPA8	Heat shock 70kDa protein 8
HSPD1	Heat shock 60kDa protein 1
HSPB1	Heat shock 27kDa protein 1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
PPC	Positive PCR control

RTC	Reverse transcription control
HGDC	Human genomic DNA contamination

Table 2.3: List of genes included in the custom PCR array used in protocol 3

Gene symbol	Official full name
VEGFA	Vascular endothelial growth factor A
CCL2	Chemokine (C-C motif) ligand 2
ANGPT2	Angiopoietin 2
ANGPT1	Angiopoietin 1
PF4	Platelet factor 4
FOXO1	Forkhead box O1
CX3CL1	Chemokine (C-X3-C motif) ligand 1
HSP90AA1	Heat shock protein 90kDa alpha class A member 1
HSP90AB1	Heat shock protein 90kDa alpha class B member 1
HSPA1B	Heat shock 70kDa protein 1B
HSPA8	Heat shock 70kDa protein 8
HSPD1	Heat shock 60kDa protein 1
HSPB1	Heat shock 27kDa protein 1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
RTC	Reverse transcription control
HGDC	Human genomic DNA contamination

Table 2.4: Serum concentrations of pro-angiogenic and inflammatory factors and the percentage of circulating pro-angiogenic cells ($CD34^+CD133^+$) before (pre) and 30 and 120 min following exposure to LBH or to a control intervention (protocol 1).

	Control (33°C)			**LBH (48°C)**		
	Pre	**30 min post**	**120 min post**	**Pre**	**30 min post**	**120 min post**
TNF-α (pg/mL)	13.2 ± 2.9	16.6 ± 3.8	16.2 ± 4.2	15.0 ± 3.3	17.5 ± 4.5	15.2 ± 3.1
IL-8 (pg/mL)	34.7 ± 9.6	39.6 ± 10.3	35.1 ± 8.7	33.4 ± 7.7	34.9 ± 8.5	38.9 ± 8.8
VEGF (pg/mL)	656.6 ± 146.8	747.9 ± 165.8	737.1 ± 149.3	648.5 ± 123.6	704.8 ± 128.5	685.6 ± 135.4
FGF2 (pg/mL)	126.0 ± 39.6	139.9 ± 37.8	137.3 ± 34.6	115.6 ± 28.8	135.2 ± 35.2	136.2 ± 38.4
G-CSF (pg/mL)	71.5 ± 22.8	81.6 ± 25.3	83.5 ± 27.1	63.1 ± 18.6	63.9 ± 16.7	70.8 ± 23.9
CCL2 (pg/mL)	666.1 ± 40.2	507.8 ± 36.0	504.1 ± 36.9	670.0 ± 48.1	511.2 ± 36.2	500.3 ± 35.9
ET-1 (pg/mL)	1.1 ± 0.06	1.5 ± 0.09	1.6 ± 0.1	1.2 ± 0.08	1.0 ± 0.08*	1.4 ± 0.08
$CD34^+CD133^+$ (% live cells)	0.2 ± 0.03	0.19 ± 0.03	0.18 ± 0.03	0.21 ± 0.05	0.21 ± 0.04	0.19 ± 0.03

Values are as means±SE. * $p<0.05$ vs. Control.

Table 2.5: Fold changes in skeletal muscle mRNA expression relative to the baseline sample of select pro-angiogenic, inflammatory and angiostatic mediators in the group exposed to LBH (n=11) and in the control group (n=12) (protocol 2). Biopsy samples were taken at baseline and 30 and 120 min following the completion of the interventions.

	Control (33°C)		**LBH (48°C)**	
	30 min	**120 min**	**30 min**	**120 min**
NOS3	1.07 ± 0.11	1.38 ± 0.25	1.16 ± 0.06	1.50 ± 0.14
PPARGC1A	1.18 ± 0.08	1.27 ± 0.16	1.32 ± 0.06	1.21 ± 0.03
MMP2	0.99 ± 0.07	1.17 ± 0.12	0.98 ± 0.07	1.07 ± 0.08
TIMP1	0.89 ± 0.09	1.18 ± 0.22	1.12 ± 0.11	0.97 ± 0.07
TEK	1.09 ± 0.11	1.30 ± 0.16	1.17 ± 0.05	1.23 ± 0.06
CXCL12	0.98 ± 0.06	0.97 ± 0.06	0.98 ± 0.03	0.93 ± 0.03
NCL	1.05 ± 0.05	1.03 ± 0.04	1.09 ± 0.04	0.96 ± 0.03
FOXO3	1.31 ± 0.06	1.34 ± 0.07	1.43 ± 0.11	1.28 ± 0.11
IL6	0.73 ± 0.14	0.66 ± 0.14	0.65 ± 0.08	0.62 ± 0.06
HSPA1A	0.80 ± 0.05	0.73 ± 0.08	1.06 ± 0.10	0.93 ± 0.08

Table 2.6: Flow profile and shear rate responses in the femoral artery to TH application in protocol 3

	Baseline		30 min		60 min		90 min		30 min post	
	C	H	C	H	C	H	C	H	C	H
d (cm)	0.66±0.02	0.68±0.02	0.69±0.02	0.70±0.02	0.68±0.02	0.71±0.02	0.69±0.02	0.71±0.02	0.68±0.02	0.72±0.03
V_{mean} (cm/s)	8.6±0.9	7.9±0.9	11.2±1.6	15.7±1.8$^{\phi}$*	12.7±1.3$^{\phi}$	17.8±1.6$^{\phi}$*	14.5±1.7$^{\phi}$	17.3±1.5$^{\phi}$*	12.8±1.6$^{\phi}$	13.1±1.4$^{\phi}$
V_{ant} (cm/s)	14.9±1.0	13.9±0.8	17.1±1.4	19.9±1.7$^{\phi}$*	17.7±1.3	21.3±1.6$^{\phi}$*	19.2±1.5$^{\phi}$	21.2±1.3$^{\phi}$	17.5±1.4	17.9±1.2$^{\phi}$
V_{ret} (cm/s)	6.3±0.7	6.07±0.6	6.0±0.7	4.3±0.3$^{\phi}$*	4.9±0.5$^{\phi}$	3.5±0.4$^{\phi}$*	4.7±0.5$^{\phi}$	3.9±0.5$^{\phi}$	4.7±0.68$^{\phi}$	4.8±0.7
BF (ml/min)	179±21	174±24	251±39	375±58*	283±39	434±57*	321±35	413±48*	286±40	322±40
SR_{mean} (s^{-1})	52.5±5.4	47.0±5.3	65.6±9.5	90.1±10.0$^{\phi}$*	75.2±7.4$^{\phi}$	100.4±8.4$^{\phi}$*	85.8±11.0$^{\phi}$	98.7±8.6$^{\phi}$	75.1±9.1$^{\phi}$	74.8±8.5$^{\phi}$
SR_{ant} (s^{-1})	90.6±6.1	82.9±5.1	100.5±8.7	114±9.2$^{\phi}$*	104.6±7.2	120±7.5$^{\phi}$*	113.1±10$^{\phi}$	121.1±7.7$^{\phi}$	102.8±8.6	101.5±7.5$^{\phi}$
SR_{ret} (s^{-1})	38.2±3.8	36.0±3.5	34.9±4.2	24.8±2.1$^{\phi}$*	29.3±3.2$^{\phi}$	19.8±2.7$^{\phi}$*	27.3±2.7$^{\phi}$	22.5±3$^{\phi}$	27.6±3.8$^{\phi}$	26.7±3.8$^{\phi}$
OSI	0.29±0.02	0.30±0.02	0.26±0.02	0.19±0.02$^{\phi}$*	0.22±0.02$^{\phi}$	0.14±0.02$^{\phi}$*	0.20±0.02$^{\phi}$	0.16±0.02$^{\phi}$*	0.21±0.03$^{\phi}$	0.21±0.03$^{\phi}$

Values are mean±SE. C, control thigh; H, heated thigh; d, diameter; V_{mean}, time-averaged mean velocity; V_{ant}, time-averaged anterograde velocity; V_{ret}, time-averaged retrograde velocity; SR_{mean}, mean shear rate; SR_{ant}, anterograde shear rate; SR_{ret}, retrograde shear rate; OSI, oscillatory shear index. $^{\phi}$ $p<0.05$ vs. Baseline; * $p<0.05$ vs. Control.

Table 2.7: Comparison of fold changes in skeletal muscle mRNA expression relative to the baseline sample of select pro-angiogenic, inflammatory and angiostatic mediators and heat shock proteins between the LBH and TH protocols.

	Protocol 2 (LBH)		Protocol 3 (TH)	
	C	**LBH**	**H-C**	**P**
VEGFA	-0.07 ± 0.08	0.29 ± 0.10	0.30 ± 0.10	0.479
CCL2	-0.42 ± 0.18	-0.13 ± 0.13	0.20 ± 0.10	0.362
ANGPT2	-0.14 ± 0.08	0.07 ± 0.06	0.33 ± 0.11	0.685
ANGPT1	0.11 ± 0.08	0.15 ± 0.04	0.30 ± 0.12	0.118
PF4	0.34 ± 0.53	2.44 ± 1.59	0.74 ± 0.55	0.286
FOXO1	0.94 ± 0.21	1.02 ± 0.25	0.01 ± 0.11	0.700
CX3CL1	-0.47 ± 0.07	-0.18 ± 0.10	0.19 ± 0.09	0.462
HSP90AA1	-0.13 ± 0.10	0.21 ± 0.05	0.33 ± 0.08	0.383
HSP90AB1	-0.06 ± 0.06	0.12 ± 0.02	0.23 ± 0.08	0.901
HSPA1B	-0.17 ± 0.06	0.21 ± 0.11	0.35 ± 0.10	0.985
HSPA8	-0.03 ± 0.04	0.16 ± 0.04	0.25 ± 0.08	0.564
HSPD1	-0.02 ± 0.06	0.17 ± 0.06	0.27 ± 0.10	0.826
HSPB1	-0.09 ± 0.11	0.08 ± 0.07	0.12 ± 0.07	0.555

Values are mean ± SEM. C, control; LBH, lower body heating; TH, unilateral thigh heating; H-C, difference in mRNA fold change between the heated thigh and the control thigh; P, p-value for the comparisons between LBH and TH.

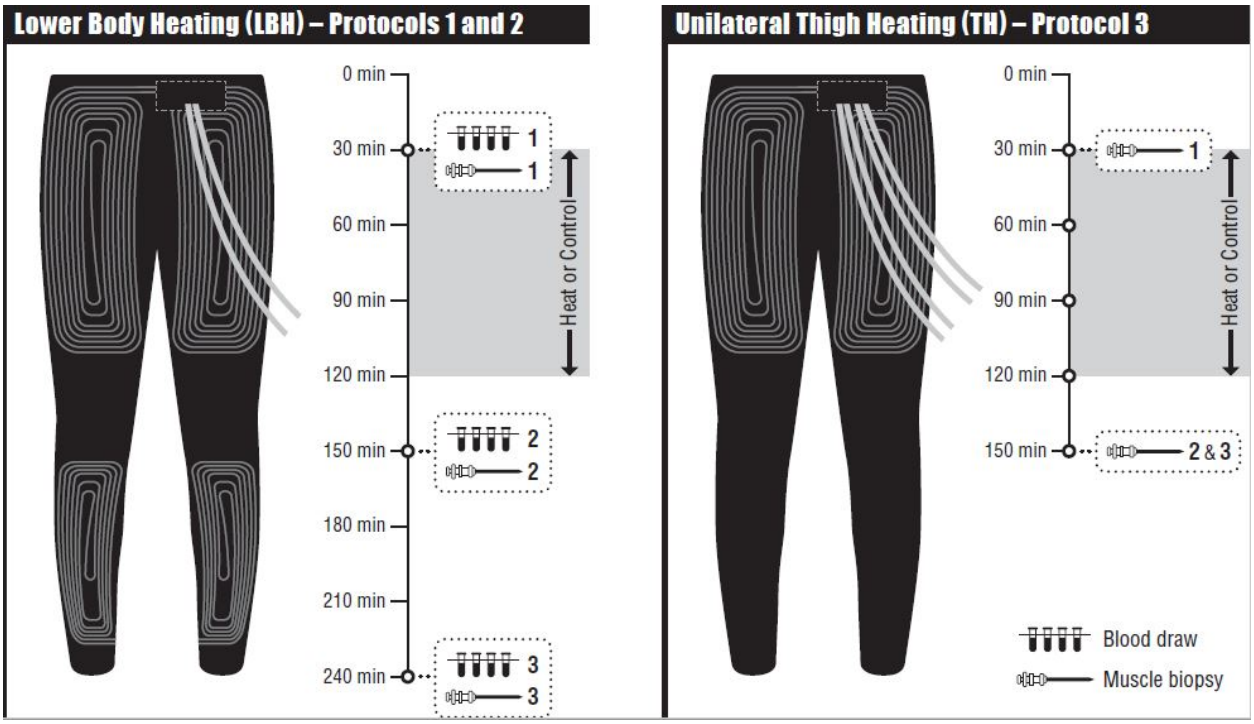

Figure 2.1: Illustration of the water-circulating garments used for heat treatment and timelines for the experimental protocols. *Left panel*: Lower body heating (LBH) was achieved by circulating hot water through tube-lined pants that covered the legs, thighs and buttocks. This approach was used on protocols 1 and 2. Blood samples (protocol 1) and muscle biopsies (protocol 2) were taken prior to and 30 and 120 min following exposure to LBH or a control intervention. *Right panel*: A custom garment with tubing around the thigh was used for local heat treatment. This garment has a separate tubing circuit for each limb, which allows for one thigh to be exposed to heat treatment while the contralateral thigh serves as a control. This approach was used for protocol 3. Muscle biopsies were taken prior to and 30 after the interventions.

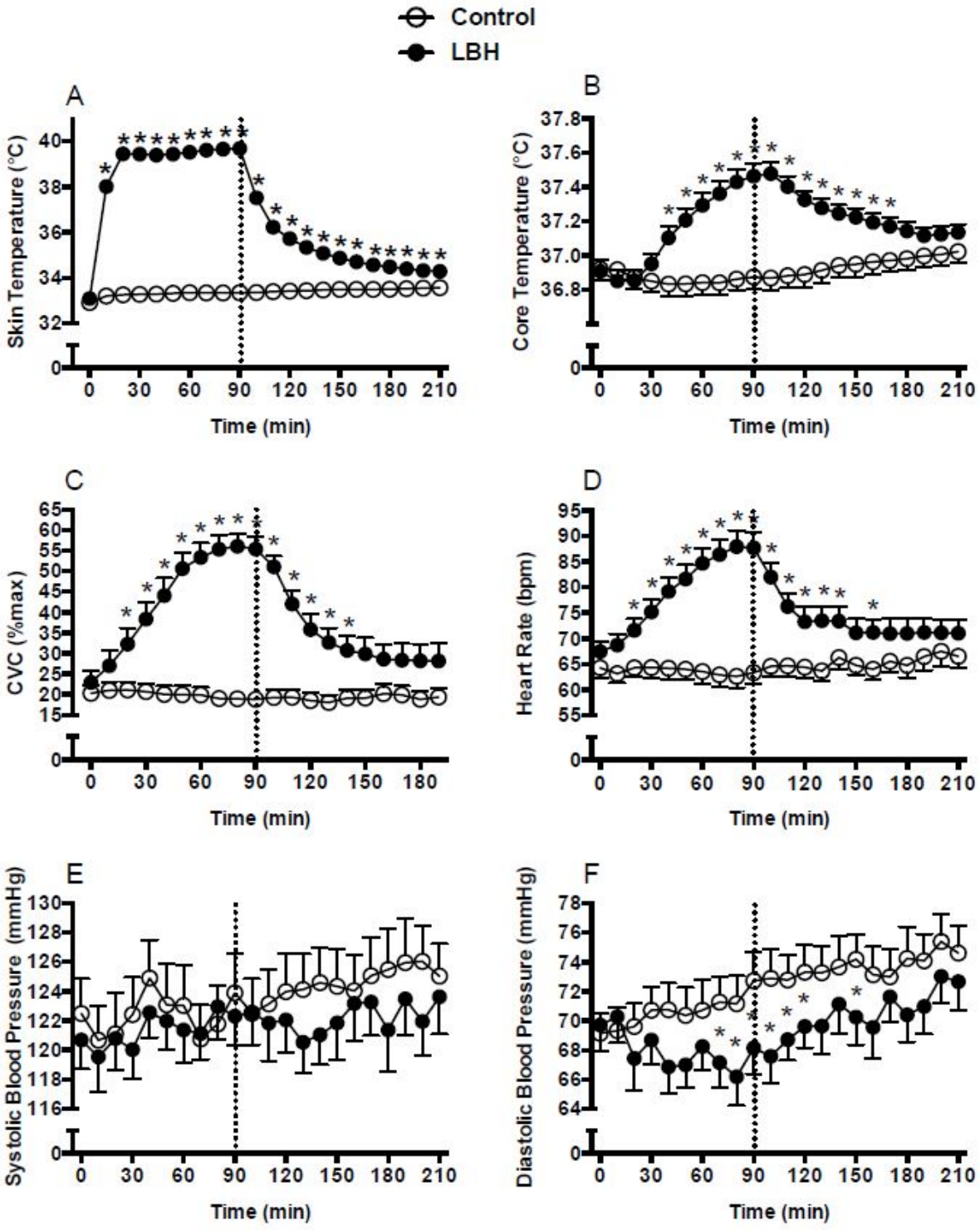

Figure 2.2: Skin (Panel A) and core temperature (Panel B), CVC (Panel C), HR (Panel D) and systolic (Panel E) and diastolic (Panel F) blood pressure before, during and after exposure to LBH (closed circles) or a control intervention (open circles) for 90 min. Dashed line indicates the end of the intervention period. * $p<0.05$ vs. Control.

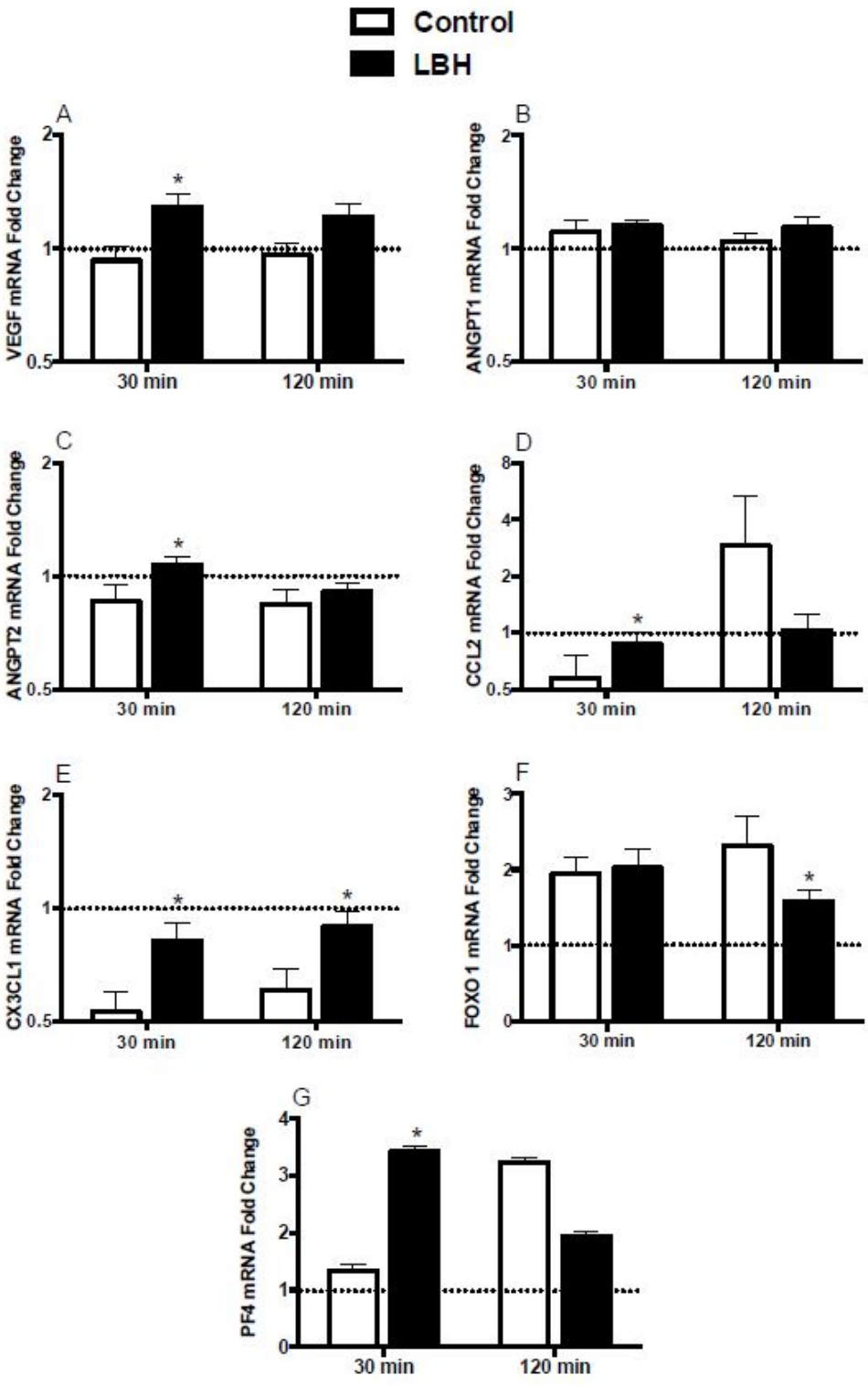

Figure 2.3: Fold changes in skeletal muscle mRNA expression relative to the baseline sample of select pro-angiogenic, inflammatory and angiostatic mediators in the group exposed to LBH (closed bars, n=11) and in the control group (open bars, n=12). Biopsy samples were taken at baseline and 30 and 120 min following the completion of the interventions. The baseline sample was assigned a value of 1 and is represented as a dashed line. * $p<0.05$ vs. Control.

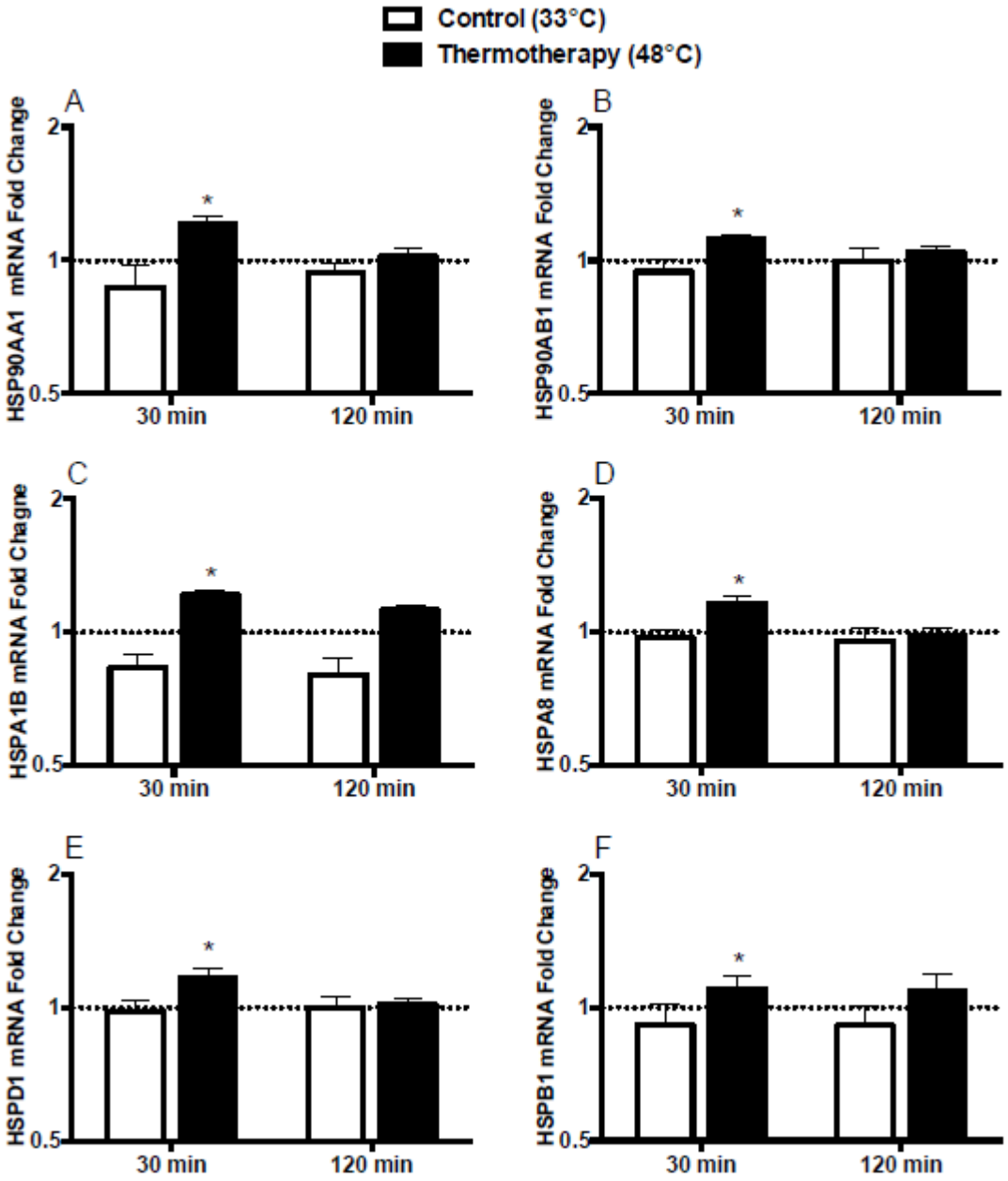

Figure 2.4: Fold changes in skeletal muscle mRNA expression relative to the baseline sample of select members of the HSP family in the group exposed to LBH (closed bars, n=11) and in the control group (open bars, n=12). Biopsy samples were taken at baseline and 30 and 120 min following the completion of the interventions. The baseline sample was assigned a value of 1 and is represented as a dashed line * $p<0.05$ vs. Control.

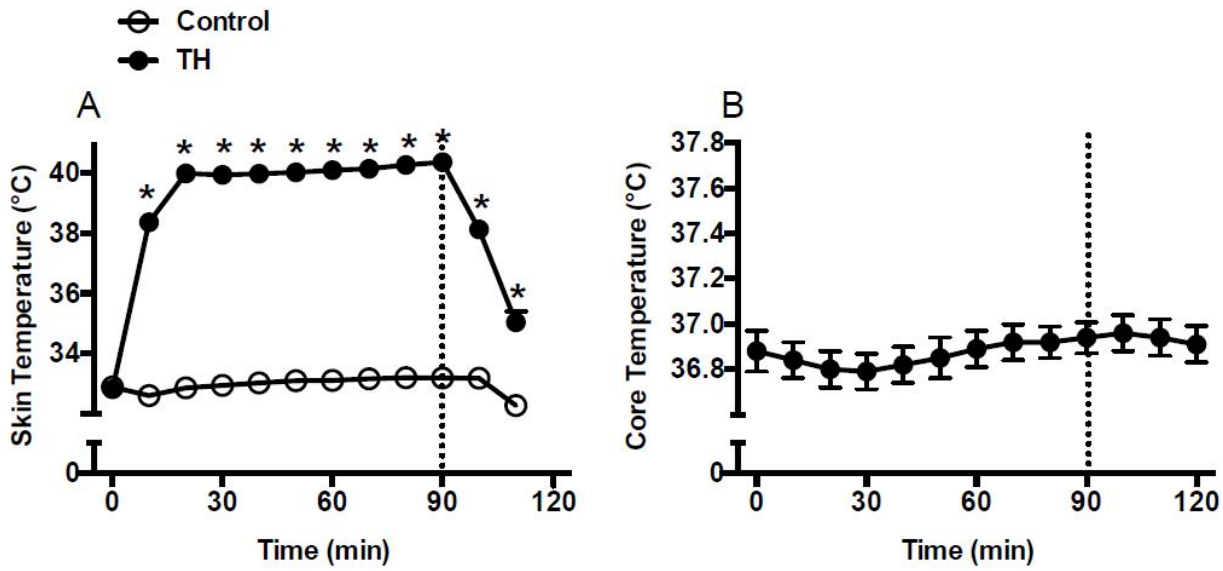

Figure 2.5: Skin temperature (Panel A) and core temperature (Panel B) before, during and after exposure to TH for 90 min. One thigh was heated (closed circles) while the opposite thigh served a control (open circles). Dashed line indicates the end of the intervention period. * $p<0.05$ vs. Control.

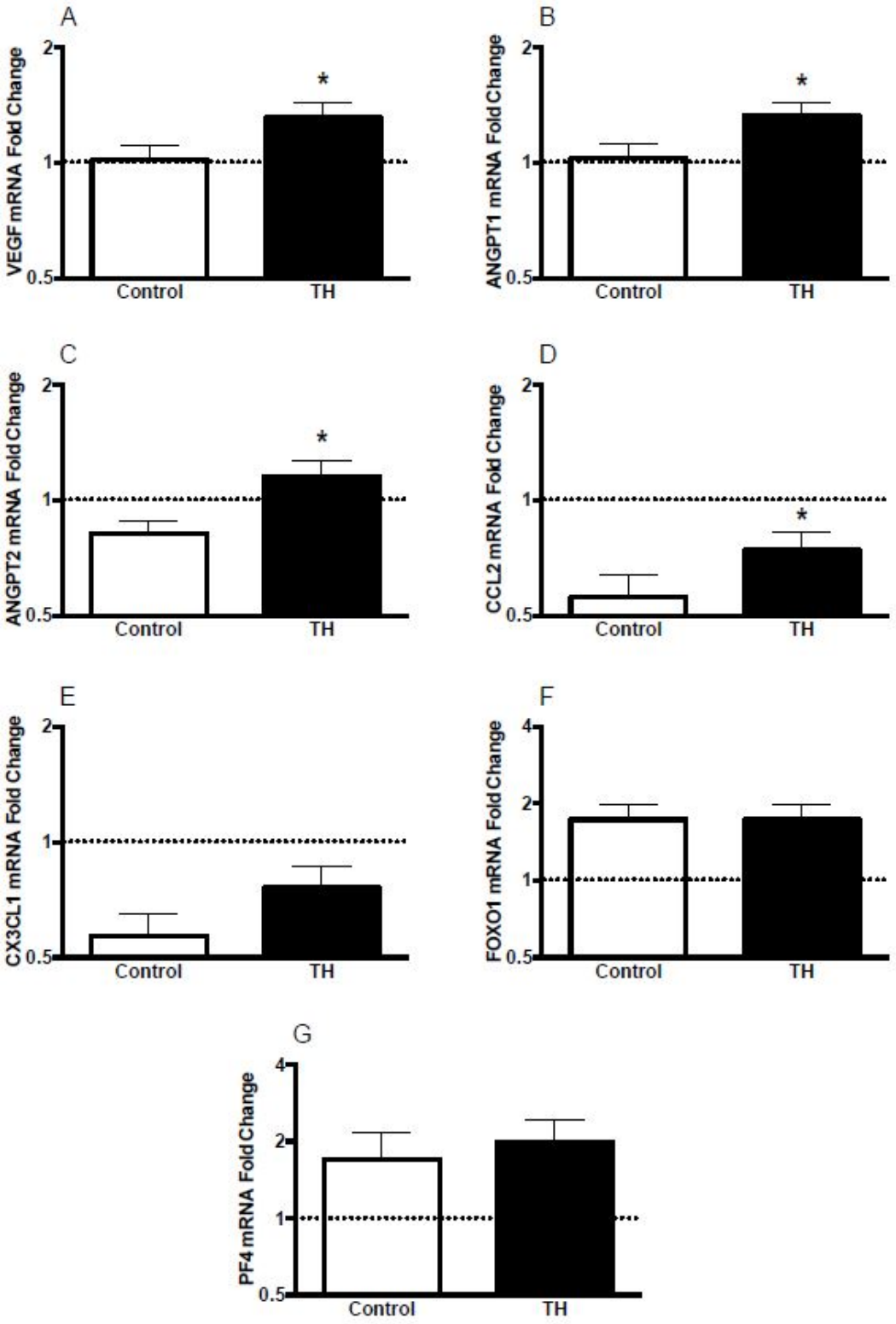

Figure 2.6: Fold changes in skeletal muscle mRNA expression relative to the baseline sample of select pro-angiogenic, inflammatory and angiostatic mediators following exposure to TH. Biopsy samples were taken at baseline and 30 min following the completion of the intervention. The baseline sample was assigned a value of 1 and is represented as a dashed line. * $p<0.05$ vs. Control.

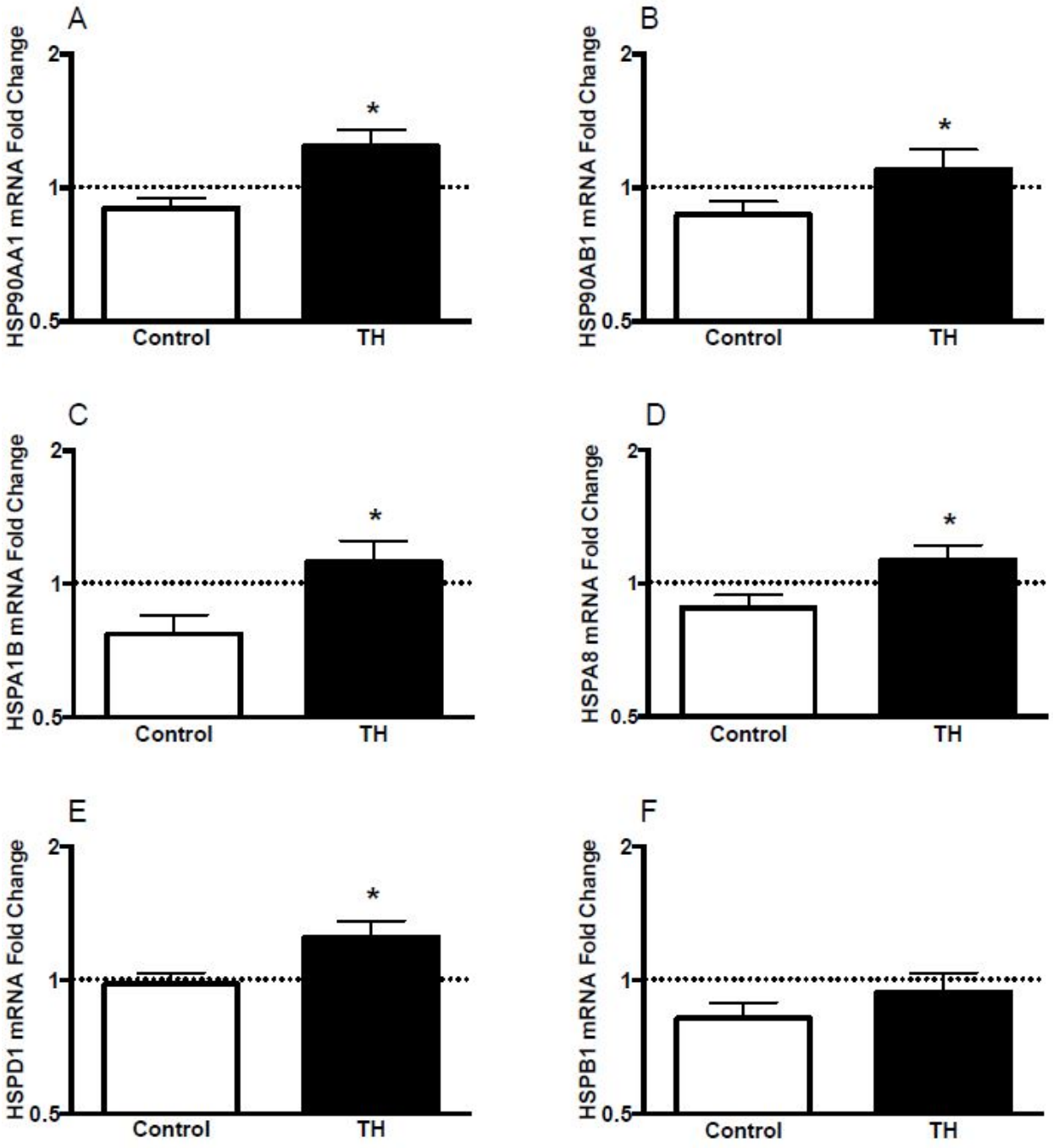

Figure 2.7: Fold changes in skeletal muscle mRNA expression relative to the baseline sample of select members of the HSP family following exposure to TH. Biopsy samples were taken at baseline and 30 min following the completion of the intervention. The baseline sample was assigned a value of 1 and is represented as a dashed line. * $p<0.05$ vs. Control.

CHAPTER 3. IMPACT OF HEAT THERAPY ON RECOVERY FOLLOWING ECCENTRIC EXERCISE IN HUMANS

Kyoungrae Kim[1], Shihuan Kuang[2], Qifan Song[3], Timothy P. Gavin[1], Bruno T. Roseguini[1]

[1]Department of Health and Kinesiology, Purdue University, West Lafayette, IN, USA

[2]Department of Animal Sciences, Purdue University, West Lafayette, IN, USA

[3]Department of Statistics, Purdue University, West Lafayette, IN, USA

Running Title: Heat therapy and skeletal muscle recovery

Correspondence:

Bruno T. Roseguini, Ph.D.

Department of Health and Kinesiology, Purdue University

800 W. Stadium Avenue, West Lafayette, IN, 47907

E-mail: brosegui@purdue.edu

Phone: 765-496-2612; Fax: 765-496-1239

Abstract

The purpose of this study was to investigate the effects of heat therapy (HT) on functional recovery, the skeletal muscle expression of angiogenic factors, the content of macrophages, and on capillarization following eccentric exercise in humans. Eleven untrained individuals (23.8 ± 0.6 yrs) performed 300 bilateral maximal eccentric contractions of the knee extensors. One randomly selected thigh was treated with five daily 90-minute sessions of HT, while the opposite thigh received a thermoneutral intervention. Peak isokinetic torque of the knee extensors was assessed at baseline and daily for 4 days, while fatigue resistance was assessed at baseline and 1 and 4 days after the eccentric exercise session. Muscle biopsies were obtained 2 weeks prior to and 1 and 5 days after the eccentric exercise bout. There were no differences between thighs in the overall recovery profile of peak torque. However, the thigh exposed to HT had greater fatigue resistance than the thigh exposed to the thermoneutral intervention. The change in mRNA expression from baseline of vascular endothelial growth factor (VEGF) was higher at day 1 in the thigh exposed to HT. Protein levels of VEGF and angiopoietin 1 (ANGPT1) were also significantly higher in the thigh treated with HT. The number of capillaries around type II fibers decreased similarly on both thighs at day 5. Exposure to HT had no impact on macrophage content. These results suggest that HT accelerates the recovery of fatigue resistance following eccentric exercise and promotes the expression of angiogenic factors in human skeletal muscle.

Key words: Heat therapy, functional recovery

New & Noteworthy

We investigated whether exposure to local heat therapy (HT) accelerates recovery following a bout of eccentric exercise in humans. When compared to a thermoneutral, control intervention, HT improved fatigue resistance of the knee extensors and enhanced the expression of the angiogenic mediators vascular endothelial growth factor (VEGF) and angiopoietin 1 (ANGPT1). These results suggest that HT hastens functional recovery and enhances the expression of regulatory factors involved in muscle repair following eccentric exercise in humans.

Introduction

Unaccustomed eccentric exercise evokes a myriad of manifestations that include reductions in muscle strength and power, soreness, swelling and reduced range of motion (14). Most of these symptoms resolve within days following minor insults, but can persist for several weeks after exposure to repeated maximal eccentric contractions (30). For example, a bout of high-force eccentric exercise with the knee extensors has been reported to cause a 40-50% strength loss (23, 38), from which full recovery can take more than 3 weeks (23). In these severe cases, the sustained impairment in muscle function can negatively impact athletic performance and reduce adherence to training regimens (15, 16).

The genesis of the prolonged impairment in muscle function following intense eccentric exercise, most notably the marked reduction in the ability to generate power, appears to stem from multiple mechanisms, including failure of excitation contraction-coupling as well as impaired metabolism (6). Recent evidence derived from animal models of exercise-induced muscle damage indicates that abnormalities in microvascular structure and function might also a play a determinant role in causing the observed performance decrements after eccentric contractions (17). In rat skeletal muscle, eccentric exercise impairs capillary hemodynamics and compromises the matching between O_2 delivery and utilization during contractions (17) and also promotes a reduction in capillarization (39). Combined, these detrimental changes can impair the ability to deliver O_2 and energetic substrates during the recovery period. As a possible compensatory response, robust changes in the expression of genes involved in capillary growth, including vascular endothelial growth factor (VEGF) and angiopoietin 1 (ANGPT1) are observed following muscle damage (51).

One important step in the reparative response following eccentric exercise-induced muscle damage is the accumulation of leukocytes in the tissue (34). Blood-borne monocytes are activated and begin to accumulate in the extracellular compartment within the muscle tissue following a bout of eccentric exercise (35). Recruitment of these immune cells, including macrophages, is coupled with increased muscle expression of cytokines and chemokines, including C-C motif chemokine ligand 2 (CCL2) and C-X3-C motif chemokine ligand 1 (CX3CL1) (35). Among other roles, it has been proposed that these inflammatory cells might contribute to the remodeling of muscle and its associated extracellular matrix to make the tissue less vulnerable to damage following subsequent insults (9).

Current therapeutic modalities employed to treat the symptoms manifested after a bout of intense eccentric exercise have proven to be largely ineffective. For example, cryotherapy, one widely popular post-exercise recovery modality, appears to delay rather than improve recovery following a bout of eccentric exercise of the elbow flexors (50) as well as arm cycling exercise (8). In animal models of muscle injury, topical icing has been shown to delay the infiltration of inflammatory cells into the damaged muscle (45, 47) and attenuate the expression of pro-angiogenic factors (45).

In sharp contrast to the observations of an impairment in muscle recovery following exposure to cryotherapy, mounting evidence indicates that heat therapy (HT) accelerates post-exercise recovery of contractile function following endurance exercise (8) and improves muscle regeneration following severe injury (19). Indeed, studies in animal models of muscle injury induced by crushing or injection of toxins revealed that repeated exposure to HT markedly improves skeletal muscle regeneration (19, 32, 44, 48). The beneficial effects of HT are thought to derive from several mechanisms, including accelerated infiltration of macrophages to the injury site (48). These findings indicate that HT might abrogate the manifestations induced by unaccustomed lengthening contractions in humans and promote faster functional recovery. Nonetheless, the majority of studies that support a favorable effect of HT have been performed in experimental models that cause extensive myofiber necrosis and require a major regenerative response. It remains unclear whether these observations hold true for the recovery following voluntary eccentric exercise performed with the lower limbs in humans.

We recently reported that a single session of HT in humans enhances the expression of genes that have shown to be critical for skeletal muscle angiogenesis and the overall remodeling response to eccentric exercise, including VEGF, ANGPT1 and several members of the heat shock protein (HSP) family (20). Based on these findings and the aforementioned studies in models of muscle injury, it is conceivable that exposure to HT promotes a local milieu that accelerates recovery following maximal eccentric exercise in humans. In the present study, young subjects performed maximal, unaccustomed eccentric knee-extension exercise with both legs and one thigh was randomly selected to receive local HT immediately after and during 4 consecutive days following the exercise bout. To gain insights into the potential mechanisms of action of HT, muscle samples were harvested to examine changes in macrophage content, skeletal muscle capillarization as well the expression of several important factors involved in muscle remodeling. We

hypothesized that, as compared to the control, thermoneutral intervention, exposure to HT would accelerate the recovery of muscle function and the accumulation of macrophages as well as enhance the expression of pro-angiogenic factors.

Methods

Participants

The Institutional Review Board at Purdue University approved all experimental procedures and verbal and written consent were obtained from all participants. Eleven young male (n=9) and female (n=2) adults (means ± SE: age 23.8 ± 0.6 years, body mass 72.4 ± 1.9 kg, height 175 ± 1.3 cm) volunteered to participate in this study. Participants were asked to fill out a medical history questionnaire. Individuals were excluded if they were obese (BMI>30 kg/m^2), used tobacco products, were diabetic, were taking any medication other than birth control, participated in leg resistance exercise in the previous 6 months, and performed endurance exercise for more than 3 days/week. Female participants were tested during the early follicular phase of their menstrual cycle (days 1-7) or during the placebo or no-pill week if they were taking oral contraceptives. All participants were asked to refrain from taking oral or topical analgesics, vitamins and antioxidants for the duration of the study.

Experimental design

Participants were asked to report to the laboratory on ten different occasions over a 3-4 week period. An overview of the experimental protocol is shown on Figure 1. On the first experimental visit, a biopsy was obtained from the vastus lateralis muscle of one randomly selected thigh to serve as the resting baseline sample. The individuals were allowed to recover for at least one week prior to the second experimental visit. The purpose of visits 2 and 3 was to familiarize the participants with the functional test on the isokinetic dynamometer as well as with the assessment of muscle soreness. These visits were separated by at least 24 hours. On visit 4, participants underwent the baseline assessment of muscle strength, fatigability and soreness as described in detailed below. At least 48 hours after visit 4, participants reported back to the laboratory to perform a bout of eccentric exercise on the isokinetic dynamometer. A 355-kcal defined formula diet (Ensure, Abbott Laboratories) was given to participants the day before the session and they were instructed to consume it at least 2 hours before the onset of the experiment.

Upon completion of the eccentric exercise bout, each thigh was assigned to receive either HT or a thermoneutral, control intervention using a counterbalanced design. The heat and control treatments began approximately 10 min after the end of the exercise bout and lasted 90 min. The treatment sessions were repeated daily for 4 consecutive days. Muscle biopsies were taken from both thighs at day 1 (24 hrs) and day 5 (120 hrs) following the eccentric exercise bout. Muscle function and perceived soreness were reassessed throughout the recovery period as outlined on Figure 1. Participants were instructed to fast for 10-11 hours before undergoing muscle biopsies and to eat a light meal prior to the other experimental visits. Participants were also asked not to perform massage or foam rolling in the thighs and to refrain from local application of ice or heat throughout the duration of the study.

Eccentric exercise

The exercise consisted of 300 maximal voluntary eccentric contractions at an angular velocity of 30º/s (20 sets, 15 repetitions per set) with the knee extensor muscles of each leg using an isokinetic dynamometer (Humac NORM, Computer Sports Medicine, Inc., Stoughton, MA, USA). As demonstrated in detail by others (26, 38), this protocol has been shown to induce several manifestations of exercise-induced muscle damage, including impaired muscle function and a pronounced elevation in local pain. After warming up on an unloaded cycle ergometer for 5 min, subjects were positioned on the chair of the isokinetic dynamometer and initially performed 75 eccentric knee extensions (5 sets of 15 repetitions with 30-s rest between sets) with one randomly selected leg. After 2-3 min of rest, subjects followed the same protocol with the other leg and the procedure was repeated 4 times. Participants were asked to resist as the lever pulled their partially extended leg from 35º of knee flexion (0º= full extension) to 105º of knee flexion, resulting in a range of motion of 70º. Consistent verbal encouragement was provided throughout the exercise bout.

Heat treatment

Local heating and the thermoneutral intervention were applied using a water-circulating garment customized to cover the thighs and buttocks (Med-Eng, Ottawa, Canada) as described previously (20). The garment consists of a network of medical grade polyvinyl chloride tubing sewn onto tight-fitting elastic fabric and connected to a water circulator. In the thigh assigned to

receive HT, water at 54-55ºC was circulated through the garment for 90 min to increase skin temperature to ~39.5-40ºC (20). In the opposite thigh, thermoneutral water (32-33ºC) was circulated to clamp skin temperature at ~33ºC for the entire duration of the protocol. The length of the treatment sessions was based on our recent report that exposure to local HT for 90 min enhances the expression of angiogenic regulators and stress management genes in human skeletal muscle (20). Skin thermocouples were taped to each thigh and allowed for skin temperature to be monitored continuously during the treatment sessions.

Muscle biopsies

Muscle biopsies (~200mg) were taken from the vastus lateralis muscle after 30 min of rest in the supine position as described previously (20). Under local anesthesia (Lidocaine hydrochloride, Hospira, Lake Forest, IL), a small incision was made in the skin and fascia followed by the insertion of a 5-mm Bergstrom biopsy needle (Pelomi Medical, Albruslund, Denmark). The biopsy specimens were promptly weighed, cleared from visible fat and connective tissue and divided into four sections. The portion designated for cryosectioning was embedded in a disposable base mold, covered with Tissue-Tek® optimal cutting temperature compound and frozen in liquid nitrogen cooled isopentane (49). The other sections were immediately frozen in liquid nitrogen and stored at -80ºC until RNA extraction or Western blot analysis. Biopsies were acquired from separate incisions, about 2-3 cm apart. Leg order for biopsy sampling was randomized for each time point. All muscle biopsies were performed between 7:30 am and 9:00 am.

Assessment of muscle strength and fatigability

Maximal knee extensor strength and fatigue resistance were assessed using an isokinetic dynamometer. Participants were familiarized with the testing procedures twice before the baseline assessment. Each session was preceded by a standardized protocol consisting of 5 min of pedaling on a cycle ergometer. Following the warm up, participants were positioned on the chair of the isokinetic dynamometer and straps were fastened at the waist, shoulders, and across the thigh to maintain a stable body position. The dynamometer settings were recorded on the first familiarization session and replicated in subsequent tests. The familiarization protocol included a set of 5-10 concentric knee extensions at 60-70% of the estimated maximal effort at an angular

velocity of 180º/s, a set of three maximal contractions at an angular velocity of 180º/s, a set of three maximal contractions at an angular velocity of 60º/s and a final set of 28 consecutive maximal contractions at 180º/s.

Maximal isokinetic strength of the knee extensors was assessed at baseline (visit 4) and daily throughout the recovery period. Fatigue resistance was assessed at baseline and 1 and 4 days after the eccentric exercise bout. Testing was performed on both legs with the order of the testing counterbalanced. On 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 1st familiarization visit. Maximal isokinetic strength was assessed at two angular velocities: 180º/s and 60º/s. Participants were asked to complete 3 maximal consecutive contractions at each angular velocity, with a resting period of 30 s between velocities and 3 min between limbs. Peak isokinetic torque was defined as the average of the two highest attained values. Once both limbs had been tested for maximal strength, participants were allowed to rest for approximately 3 min and were then asked to perform a bout consisting of 28 consecutive maximal contractions at 180º/s. A resting period of 10 min was allowed between limbs. The total work performed during the bout was computed and used as a measure of fatigue resistance of the knee extensors. The reliability of muscle performance assessment was determined by comparing the results of the 2nd familiarization visit (visit 3) and the baseline testing visit (visit 4). Both measures of muscle strength and fatigue resistance were found to be highly reproducible (retest correlations: 1) peak toque at 180º/s: 0.91, 2) peak toque at 60º/s: 0.92, 3) total work completed during the fatigue bout: 0.97).

Perceived muscle soreness

Participants were asked to evaluate knee extensor muscle soreness on a 10-cm visual analog scale (0 = no soreness, 10 = extreme soreness) after stepping on and off a 40 cm (female) or 45 cm (male) chair three times.

RNA extraction

Total RNA from the skeletal muscle biopsies was prepared using the TRIzol Reagent (Invitrogen, Life Technologies, Carlsbad, CA) and quantified spectrophotometrically (Nanodrop 3000, ThermoFisher), as described previously (20). The quality and integrity (RIN of 8.2 ± 0.1) of

extracted RNA (276.9 ± 26.4 ng/µl) was evaluated using an RNA 6000 Nano LabChip kit on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). cDNA was prepared using the RT^2 First Strand Kit following the manufacturer's instructions (Qiagen, Valencia, CA).

Skeletal muscle gene expression

The expression of 9 select genes related to angiogenesis, myogenesis, inflammation and stress management was determined using a custom RT^2 Profiler PCR array kit (Qiagen, Valencia, CA) and the Roche LightCycler 480 PCR System (Roche Diagnostostics, Indianapolis, IN). Duplicate samples from each subject (baseline, day 1 and day 5 post eccentric exercise) were loaded in the same array plate. The list of genes analyzed is shown in Table 1 and included the housekeeping gene 18S ribosomoal RNA (18S rRNA) as well as a reverse-transcription control, a positive PCR control and a genomic DNA control. Data was analyzed using the GeneGlove Data Analyses Center (Qiagen, Valencia, CA). 18S rRNA was used as the internal reference gene and proved stable across all time points. The comparative Ct method was used to calculate the changes in gene expression of each target mRNA relative to the baseline sample (22).

Protein extraction

Frozen muscle samples (~30 mg) were homogenized in ice-cold homogenization buffer containing 0.5M Tris-HCl, pH 7.4, 1.5M NaCl, 2.5 % deoxycholic acid, 10% NP-40 and 10mM EDTA (RIPA Lysis Buffer, EMD Milipore) with freshly added protease inhibitor cocktail (P8340, Sigma-Aldrich) at a 1:15 dilution of wet muscle weight using a bead mill homogenizer (BEAD RUPTOR12, 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, USA). All samples were subsequently diluted with 1x phosphate-buffered solution (1.8 µg/µL) and subsequently mixed with either reducing sample buffer (4x Laemmli sample buffer with 10% 2-Mercaptoethanol) or non-reducing sample buffer (4x Laemmli sample buffer). Afterwards, samples were heated to 95ºC for 5 min, divided into small aliquots and stored at -80ºC.

Western Blot Analysis

25 µg of proteins were separated by SDS-PAGE on precast Stain Free 4-15% gels (Bio-Rad, CA, USA) and transferred to polyvinylidene fluoride (PVDF) membranes using the Trans-Blot® Turbo transfer system (Bio-Rad, CA, USA). Membranes were subsequently blocked with 5% non-fat milk in 1x TBST (1% tween 20) solution for 1 hr at room temperature and incubated overnight at 4°C with primary antibodies diluted in blocking buffer. Details of the primary antibodies and recombinant proteins are provided in Table 2. The membranes were washed with 1x TBST at room temperature for 3 x 10 min, incubated with horseradish peroxidase-conjugated secondary antibodies diluted in 1x TBST for 1 h at room temperature and were then washed with 1x TBST at least 3 x 10 min before being exposed to an enhanced chemiluminescent solution (Clarity Western ECL, Bio-Rad, USA) for 5 min. Membranes were visualized using a densitometer (ChemiDoc Touch Imaging System, Bio-Rad, USA) and band densities were determined using image-analysis software (Image Lab V5.0, Bio-Rad, USA). PageRuler Prestained Protein Ladder (Thermo Fisher, USA) was used as a molecular weight marker. Control for equal loading was performed using the stain-free technology and total protein normalization was used to calculate changes in the expression of each target protein relative to the baseline sample (12).

Immunohistochemistry

Transverse serial sections (10 µm) of muscle biopsy samples were cut using a Leica CM1850 cryostat (Leica, Wetzlar, Germany) at -25°C, mounted on frosted microscope slides (Thermo Scientific, NH, USA), air-dried for 0.5-1h 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. Following 2 x 3 min washes with 1x PBS, the slides were incubated with blocking buffer (5% goat serum, 2% bovine serum albumin, 0.1% Triton X-100 and 0.1% azide in PBS) for 1 hr at room temperature (52). Thereafter, sections were incubated with primary antibodies diluted in blocking buffer for 3 hrs at room temperature. Macrophages were stained using an antibody for CD68 (mouse IgG2b; 1:100; Cat. No. MAB20401; R&D system), while laminin (rabbit IgG1; 1:500; Cat No. ab11575; Abcam) was added for distinction of the myofiber sarcolemma. Identification of fiber type-specific capillaries was performed using antibodies against CD31 (mouse IgG1; 1:100; Cat No. 550300; BD Biosciences), dystrophin (rabbit IgG1; 1:100; Cat. No. ab15277; Abcam) and myosin heavy chain type I (MHC-I) (mouse IgG2b;

supernatant 1:100; Cat. No. BA-D5; DSHB). After 2 x 5 min washes with 1x PBS, sections were stained with appropriate secondary antibodies (Alexa 350 goat anti-rabbit IgG; 1:500; Cat. No. A-11069, Alexa 488 goat anti-rabbit IgG; 1:1000; Cat. No. A-11008, Alexa 488 goat anti-mouse IgG 2b; 1:1000; Cat. No. A-21141, and Alexa 568 goat anti-mouse IgG1; 1:1000; Cat. No. A-21124, Thermo Fisher Scientific), diluted in 1x PBS for 1 hr at room temperature. Nuclei were labeled with 4', 6'-diamidino-2-phenylindole (DAPI). Following 4 x 5 min washes, slides were briefly dried and mounted using fluorescent mounting medium (Dako, CA, USA) and the edges were sealed with nail polish (Sally Hansen Hard as Nails, NY, USA). Negative controls for the primary antibodies against CD31 and CD68 were used to ensure specificity of staining. Slides were viewed at ×20 magnification using an Olympus BX53 fluorescence microscope equipped with an Olympus DP72 digital camera and CellSens Dimension software. The entire specimen cross section was initially selected using the stage navigator. The multi-channel image was then acquired and two images from each channel were merged using Image J software (National Institutes of Health, USA). Histological analysis was not performed in 3 out of 55 samples due to insufficient muscle yield.

Analysis of immunofluorescence images

Analyses of immunofluorescence images were carried out using Adobe Photoshop CC 2015. For the quantification of fiber type-specific capillarization, all internal fibers (not bordering on a fascicle) in a cross section were initially counted (an average of 173 ± 95 fibers for type I and 217 ± 121 for type II muscle fibers). A total of 50 type I and 50 type II muscle fibers were then randomly selected for analysis (37). All capillaries within a distance of 5 μm from each fiber were counted for the determination of the number of capillaries around each fiber (CAF) (55). To evaluate the distribution and perform the quantification of macrophages, an average of 736 ± 297 fibers was analyzed. Macrophages were identified when the fluorescent signal for CD68 clearly surrounded (11) or entirely covered a nucleus. All immunofluorescent images were blinded for both group and time point prior to analysis.

Statistical analysis:

All statistical analyses were conducted using SAS (Version 9.4; SAS Institute) with results expressed as means ± SE. The Kolmogorov-Smirnov test was used to assess the distribution of the

data. Data exhibiting skewed distribution were log-transformed before statistical analysis. A two-way repeated measures ANOVA was employed to compare the changes in skeletal muscle performance and soreness, gene and protein expression responses and changes in the content of $CD68^+$ immune-reactive macrophages and CAF between the leg exposed to HT and the control leg. Post-hoc analysis (Tukey) was performed when appropriate. For all analyses, $P < 0.05$ was considered statistically significant.

Results

Performance during eccentric exercise

Figure 2 depicts the amount of work completed during each of the 20 sets of eccentric contractions by the thigh that was later assigned to receive HT and by the thigh that was subsequently allocated to the thermoneutral, control treatment. On average, the thigh randomized to receive HT performed slightly more work (4.6%) than opposite thigh (control: 1649 ± 108 J vs. HT: 1724 ± 115 J). All participants were right-leg dominant (based on kicking preference). Six participants were assigned to receive HT on their dominant leg and 5 individuals had their dominant leg treated with the control regimen.

Changes in muscle function in response to muscle damage and recovery

The responses of isokinetic peak torque, total work completed during the fatigue bout and perceived muscle soreness are displayed on Figures 3-5. There were no significant differences between thighs for baseline measures of isokinetic peak torque at 180º/s (control: 139 ± 2.3 Nm vs. HT: 143 ± 1.9 Nm, p=0.29), 60º/s (control: 181 ± 3.2 Nm vs. HT: 181 ± 2.4 Nm, p=0.37) and total work amount completed during the fatigue trial (control leg: 3531 ± 56 J vs. HT: 3575 ± 60 J, p=0.60). Peak torque decreased on average by ~45% in the first two days following the eccentric exercise protocol and began to recover progressively starting at day 3 (Figure 3). There were no differences between thighs in the overall recovery profile of peak torque. Fatigue resistance, as determined by the total work amount completed during 28 consecutive maximal contractions at 180º/s, decreased by approximately 20% at day 1 following the completion of eccentric exercise bout and remained below baseline values at day 4 (Figure 4). The thigh exposed to HT had significantly greater fatigue resistance (i.e. lower reduction in total work relative to baseline) when compared to the control thigh (p=0.02, main effect). Perceived muscle soreness rose sharply after

the eccentric exercise bout, reaching peak values at day 2 (Figure 5). Exposure to HT tended to decrease the magnitude of muscle soreness relative to the control treatment ($p=0.053$).

Gene expression

Changes in gene expression from baseline for select angiogenic, myogenic, inflammatory and stress management genes are shown on Figure 6. At day 1, the mRNA expression of VEGF (Control: 0.79 ± 0.10 vs. HT: 1.24 ± 0.12, $p<0.05$) was significantly higher in the thigh exposed to HT as compared to the control thigh. Conversely, at day 5, the mRNA expression of ANGPT1 (Control: 1.02 ± 0.13 vs. HT: 0.78 ± 0.12, $p<0.05$) was significantly lower in the thigh exposed to HT. The mRNA expression of CCL2 also was significantly lower in the thigh exposed to HT ($p=0.01$, main effect).

Protein expression

Figure 7 displays the changes in the protein levels of select angiogenic, myogenic, inflammatory and stress management factors relative to the baseline sample. A significant increase in the expression of chemokine CX3CL1 was observed in the thigh exposed to HT as compared to the control thigh on day 1 (Control: 0.87 ± 0.07 vs. HT: 1.21 ± 0.13, $p<0.05$). The expression of VEGF ($p=0.005$, main effect), ANGPT1 ($p=0.045$, main effect) and CCL2 ($p=0.02$, main effect) was higher in the thigh treated with HT as compared to the control thigh.

Macrophage content

Skeletal muscle cross sections were analyzed for the presence of $CD68^+$ macrophages using immunohistochemistry (Figure 8). The majority of $CD68^+$ immune-reactive macrophages were detected in between fibers (Figure 8, panel A). Intracellular staining for CD68 was only detected in very few fibers. An increasing trend in the number of $CD68^+$ cells per 100 muscle fibers was noted after exposure to eccentric exercise ($p=0.071$, Figure 8, panel B). On average, macrophage content increased by 44% on day 1 and by 130% on day 5. There was no treatment effect or a treatment by time interaction for this variable.

Skeletal muscle capillarization

One representative image of a muscle cross-section stained for MHC-I, CD31 and dystrophin is displayed on Figure 9 (Panel A). There was no treatment effect or a treatment by time interaction for CAF. A significant decrease in CAF was observed at day 5 following the eccentric exercise bout in type II fibers ($p<0.05$).

Discussion

The main findings of the present study are that when compared to a thermoneutral intervention, exposure to HT following a bout of maximal eccentric exercise: 1) accelerated the recovery of fatigue resistance, 2) increased the mRNA expression of VEGF at day 1, 3) increased protein levels of CX3CL1 at day 1, 4) increased protein levels of pro-angiogenic factors VEGF and ANGPT1 and chemokine CCL2. Conversely, treatment with HT had no impact on the recovery of muscle strength, skeletal muscle macrophage content and capillarization as well as on HSP expression.

The persistent decline in muscle function following unaccustomed eccentric exercise, most notably in the ability of the muscles to generate power, has detrimental consequences for athletic performance and adherence to training regimens (6). A marked decline in power output and reduced work capacity during maximal dynamic exercise is commonly observed after eccentric exercise in humans (5, 41, 43). In agreement with these reports, we observed that the total work completed during 28 consecutive maximal contractions at 180º/s was reduced by ~20% on the day after a bout of eccentric exercise and was not fully restored after 4 days (Figure 4). The genesis of this prolonged reduction in muscle work capacity is multifactorial, but it is recognized that changes in metabolic function play an important role (6). For instance, muscle glycogen resynthesis is impaired after eccentric exercise, possibly because of reduced glucose uptake (1, 18) as well as reductions in GLUT-4 content (3). Consequently, muscles exposed to eccentric exercise have to work at a higher relative workload during a subsequent bout of concentric exercise, resulting in increased glycogen utilization and decreased endurance (2). One important finding of the present study is that work capacity was greater in the thigh that received HT as compared to the control thigh (Figure 4). Although we did not investigate the specific mechanisms behind this effect, it is conceivable that HT accelerated the resynthesis of glycogen, resulting in greater dynamic power output during the maximal work capacity test. Two pieces of evidence lend strong support to this

hypothesis. Slivka and co-workers first demonstrated that local muscle heating during recovery from a bout of exhaustive cycling exercise increased the rate of glycogen resynthesis in active male subjects (46). More recently, Cheng and co-workers showed that glycogen resynthesis following fatiguing stimulation in mouse intact single muscle fibers was accelerated by increasing muscle temperature, which, in turn, led to improved fatigue resistance (8). It is important to emphasize, nonetheless, that these studies focused on the recovery following exhaustive endurance exercise and it remains to be determined whether HT accelerates glycogen resynthesis following maximal eccentric exercise.

The finding that perceived soreness tended to decrease in the thigh exposed to HT as compared to the thigh received the control treatment is in line with a growing number of studies that indicate that local heating early after vigorous eccentric exercise reduces local pain. For example, Mayer and co-workers reported that treatment with a heat wrap starting 4 hours after a bout of eccentric lumbar extension exercise was more effective at reducing local pain than cryotherapy (24). Petrofsky and co-workers recently showed that a heat wrap placed over the quadriceps immediately after a bout of eccentric exercise significantly reduced perceived pain when compared to a control intervention (36). This hypoalgesic effect of HT is thought to be caused in part by heat-induced increases in blood flow (13) and the consequent accelerated removal of factors that sensitize muscle nociceptors. One view is that mechanical hyperalgesia following eccentric exercise is caused by the production by muscle fibers and/or muscle satellite cells of nerve growth factor (NGF) and glial cell line-derived neurothropic factor (GDNF) (27). Of note, treatment with hot packs has been shown to reduce the muscle content of NGF and substantially reduced physical-inactivity-induced mechanical hyperalgesia in rats (29).

The beneficial effects of HT on skeletal muscle recovery following damage have also been proposed to stem from the accelerated recruitment of inflammatory cells to the injury site (48). A recent study in a model of muscle crush injury in rats revealed that HT applied immediately after injury speeded the infiltration of M1 macrophages that express the cell surface molecule CD68 (48). These findings are relevant because M1 macrophages have been shown to enhance muscle regeneration in rodents by interacting with proliferating satellite cells (40) and reducing fibrosis (31). An increase in the number of $CD68^{+}$ immune-reactive macrophages has been consistently observed in the endomysium and perimysium following a bout of lengthening contractions in humans (4, 10, 34). One prevailing view is that these cells are likely recruited to repair tears within

the extracellular matrix (10). Consistent with previous reports, we observed that $CD68^+$ macrophages were predominantly located in the extracellular compartment after exposure to exercise (Figure 8, panel A). Our hypothesis was that, based on the aforementioned observation in a rodent model of muscle injury (48), HT would speed the arrival and consequently increase the content of $CD68^+$ macrophages. Chemoattractants produced by muscle cells, including CX3CL1and CCL2, appear to be critical for the recruitment of inflammatory cells to injured skeletal muscle (7). Of note, treatment with HT evoked increased protein expression of CX3CL1 and CCL2 during recovery (Figure 7, panels C and D). However, in spite of the changes in CCL2 and CX3CL1 levels, exposure to HT did not impact the dynamics and magnitude of macrophage recruitment.

We previously reported that a single 90-min session of HT enhances the skeletal muscle mRNA expression of pro-angiogenic factors VEGF and ANGPT1 in humans (20). This observation prompted us to test the hypothesis that HT creates a milieu that favors capillary growth and mitigates the detrimental effects of eccentric exercise on the muscle microcirculation. Eccentric exercise has been shown to induce pronounced alterations in the skeletal muscle microcirculation in rats and in humans. Kano and co-workers first reported that downhill running, which forces eccentric contractions within the rat spinotrapezius, caused a significant increase in the proportion of capillaries that do not support continuous red blood cell flow (17). The decrease of microvascular oxygen pressure during electrically stimulated contractions was also accelerated in muscles of animals subjected to eccentric exercise, which is compatible with a slowed exercise hyperemic response to muscle contractions (17). Along the same lines, Larsen and co-workers recently demonstrated that a single bout of eccentric contractions of the dorsiflexor muscles slows microvascular reactivity during brief contractions in humans (21). In addition to causing abnormalities in vascular function, there is evidence that eccentric exercise negatively impacts microvascular structure. Rizo-Roca and co-workers reported that a double session of strenuous eccentric exercise in trained rats caused a marked decline in capillary density and capillary-to-fiber ratio in the soleus muscle (39). Of note, the reduction in capillarization was evident within 1-3 days following the exercise bouts (39). In humans, Yu and co-workers also reported that a single bout of downstairs running induced capillary regression in some, but not all, individuals during the recovery period (56). In the present study, we observed a decline in the number of capillaries around type II muscle fibers 5 days after the eccentric bout (Figure 9, and C), but contrary to our

predictions, exposure to HT did not prevent this effect. However, HT did induce increases in the expression of VEGF and ANGPT1 and it is possible that these factors might facilitate capillary growth later in the recovery period.

A hallmark response to strenuous exercise is the up-regulation in skeletal muscle of stress management proteins, most notably the members of the heat shock protein (HSP) family. These molecular chaperones are thought to be fundamental in facilitating the cellular remodeling processes evoked by muscle injury as well as mediating the adaptations to exercise training (28). For example, overexpression of HSP70 enhances recovery from damage induced by lengthening contractions in mice (25), while ablation of this protein severely impairs muscle regeneration following injury caused by cardiotoxin injection or muscle reloading following a period of muscle disuse injury (42). Based on our recent observation that HT elicits increased expression of several members of the HSP family (20), we had anticipated that HT would augment the response of HSP90 and HSP70 to eccentric exercise. While we did observe the expected increase in the expression of these factors following eccentric exercise, these changes were comparable between the muscles exposed to HT and the control treatment. It is tempting to speculate that the potent stress response triggered by the maximal bout of eccentric exercise prevented the detection of an additive effect induced by HT. Further studies are necessary to examine whether this same pattern remains later in the recovery process (i.e. beyond 5 days), when the stress response is resolved and HSP levels begin to return to baseline levels (28).

Limitations

One particular challenge in the design of studies aimed at assessing the impact of HT on recovery following exercise is that it is not possible to blind subjects to the exposure to heat. Although participants in the present study were not informed of the study hypothesis and a control intervention was used for comparison, we did not examine the potential influence of the placebo effect on the observed responses. Accumulating evidence indicates that therapeutic effects of popular recovery modalities, such as cryotherapy, stem, at least partially, from a placebo effect (53, 54). These important observations emphasize the need for future studies on the effects of HT to implement an effective placebo controlled design.

Recovery from intense eccentric exercise entails a complex, well-orchestrated response that may persist for several weeks. Insights into the mechanisms underlying the beneficial effects

of HT were limited in the present study because muscle biopsies were harvested only in the immediate (day 1) and sub-acute (day 5) phases of recovery. Indeed, some of the manifestations of exercise-induced muscle damage, including the decline in force and power output, increased soreness and reduced capillarization, were not fully resolved by day 5. Future studies should examine the impact of repeated local HT on the later phases of recovery.

Perspectives and significance

Our results show that exposure to HT immediately after and for 4 consecutive days following a maximal bout of eccentric exercise in humans hastens recovery of fatigue resistance and tends to reduce perceived soreness. These findings are in line with a growing body of literature supporting a beneficial impact of HT on recovery following non-injurious exercise (8) as well as severe muscle injuries (19, 32, 33, 44, 48). Additional studies are warranted to determine whether repeated exposure to local HT amplifies the skeletal muscle adaptations to exercise training in humans.

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Additional information section

There are no conflicts of interests.

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Table 3.1: List of genes included in the custom PCR array

Gene symbol	Official full name
HSPA1A	Heat shock 70kDa protein 1A
HSPA1B	Heat shock 70kDa protein 1B
HSP90AA1	Heat shock protein 90kDa alpha class A member 1
HSP90AB1	Heat shock protein 90kDa alpha class B member 1
VEGFA	Vascular endothelial growth factor A
ANGPT1	Angiopoietin 1
CCL2	Chemokine (C-C motif) ligand 2
CX3CL1	Chemokine (C-X3-C motif) ligand 1
MSTN	Myostatin
RRN18S	18S ribosomal RNA
RTC	Reverse transcription control
HGDC	Human genomic DNA contamination

Table 3.2: List of antibodies used for western blotting

Antigen	Primary antibody	Secondary antibody	Recombinant protein
Heat shock protein 70s	Bio-Rad (VMA00042)	Bio-Rad (anti-mouse STAR207P)	Bio-Rad (VMA00042KT)
~ 70 kDa			
Reducing	1:2000	1:10000	
Heat shock protein 90A	Bio-Rad (VMA00081)	Bio-Rad (anti-mouse STAR207P)	Bio-Rad (VMA00081KT)
~ 90 kDa			
Reducing	1:500	1:10000	
Heat shock protein 90B	Bio-Rad (VMA00082)	Bio-Rad (anti-mouse STAR207P)	Bio-Rad (VMA00082KT)
~ 90 kDa			
Reducing	1:2000	1:10000	
VEGF165	R&D system (AF-293-NA)	R&D system (anti-goat HAF017)	R&D system (293-VE-010)
~ 39 kDa			
Non-reducing	1:200	1:10000	
Angiopoietin 1	R&D system (AF923)	R&D system (anti-goat HAF017)	R&D system (923-AN-025)
~70 kDa			
Reducing	1:1000	1:10000	
Myostatin	R&D system (AF788)	R&D system (anti-goat HAF017)	R&D system (788-G8-010)
~ 26 kDa			
Non-reducing	1:2500	1:10000	
CX3CL1	R&D system (AF365)	R&D system (anti-goat HAF017)	R&D system (365-FR-025)
~ 90 kDa			
Reducing	1:2500	1:10000	
CCL2	R&D system (MAB679-100)	R&D system (anti-goat HAF017)	R&D system (279-MC-010)
~ 10 kDa			
Non-reducing	1:1000	1:10000	

	Baseline	Muscle injury	Post-injury				
		Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Muscle soreness	x	x	x	x	x	x	x
Muscle biopsy	x		x				x
Muscle strength	x		x	x	x	x	
Muscle fatigability	x		x			x	
Eccentric exercise		x					
HT/Control		x	x	x	x	x	

Figure 3.1: Schematic overview of the study protocol.

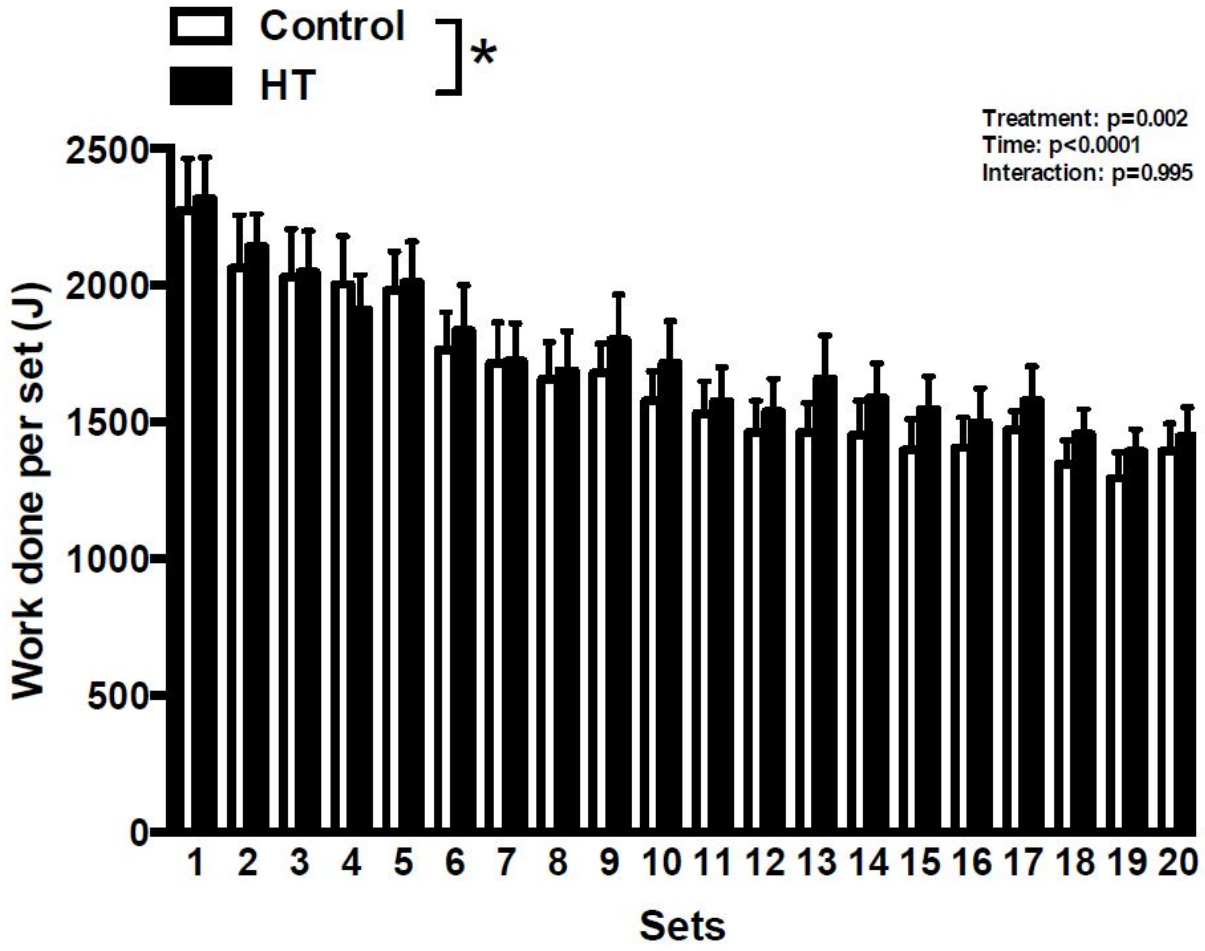


Figure 3.2: Performance during eccentric exercise. Participants (n=11) performed 300 bilateral eccentric contractions of knee extensors (20 sets of 15 reps). Data were analyzed using a two-way repeated measures ANOVA. Values are means ± SE. * Main effect for treatment (p=0.002).

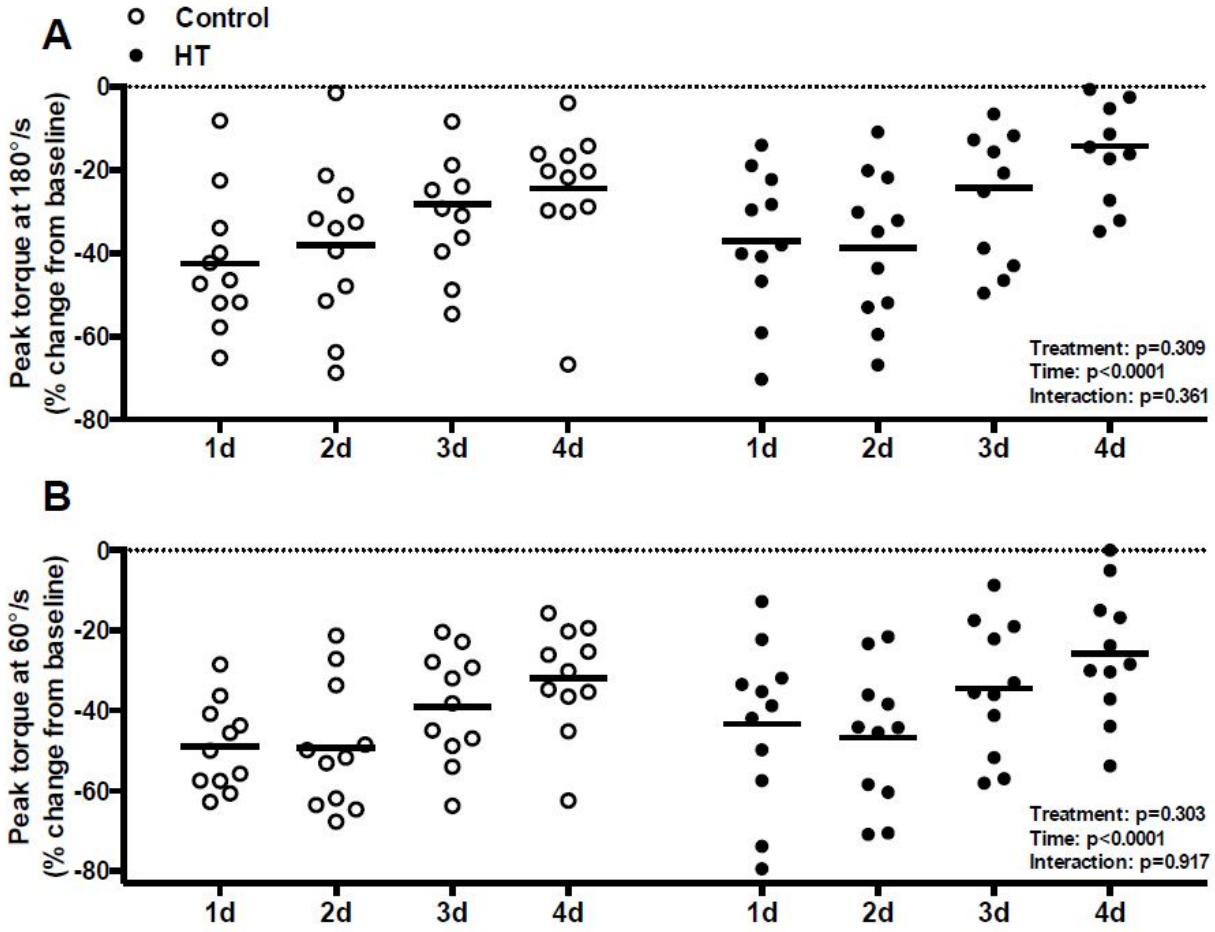


Figure 3.3: Individual and group mean changes in peak torque during knee extensions at 180º/s (panel A) and 60º/s (panel B) following the eccentric exercise bout in the thigh exposed to the control, thermoneutral intervention (open symbols) and the thigh treated with HT (closed symbols). Data were analyzed using a two-way repeated measures ANOVA. Values are means ± SE.

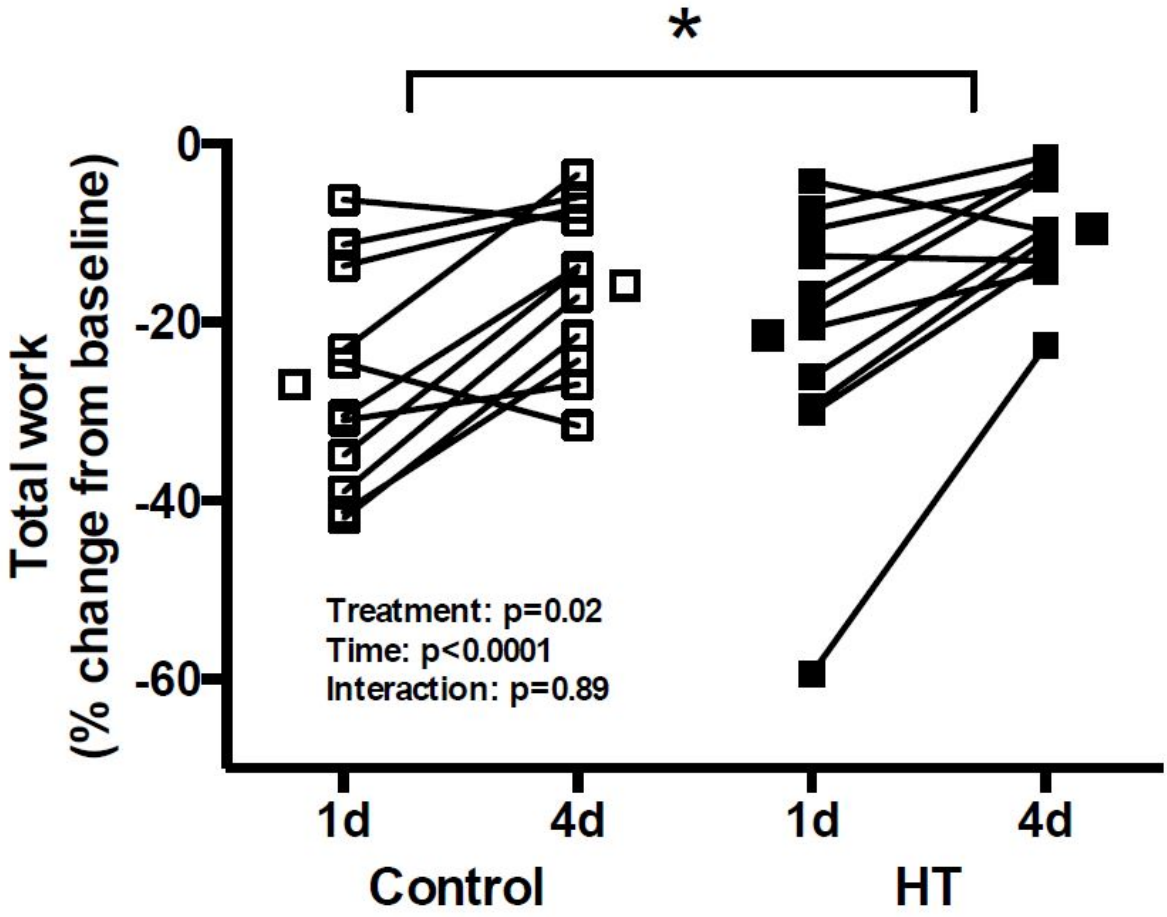


Figure 3.4: Individual and group mean changes in fatigue resistance following the eccentric exercise bout in the thigh exposed to the control, thermoneutral intervention (open symbols) and the thigh treated with HT (closed symbols). Data were analyzed using a two-way repeated measures ANOVA. Values are means ± SE. * Main effect for treatment (p=0.02).

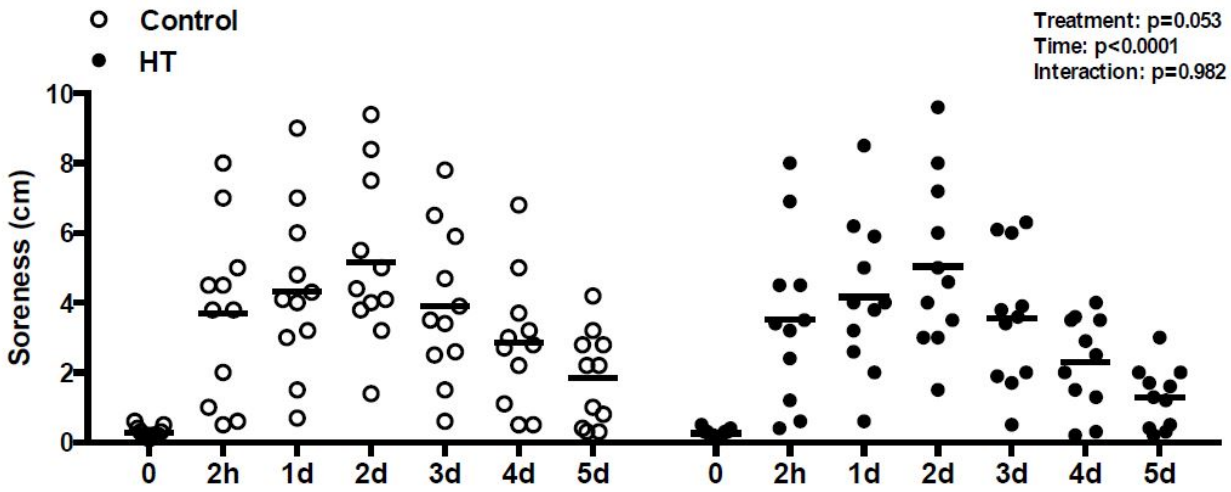


Figure 3.5: Individual and group mean changes in perceived muscle soreness following the eccentric exercise bout in the thigh exposed to the control, thermoneutral intervention (open symbols) and the thigh treated with HT (closed symbols). Data were analyzed using a two-way repeated measures ANOVA. Values are means ± SE.

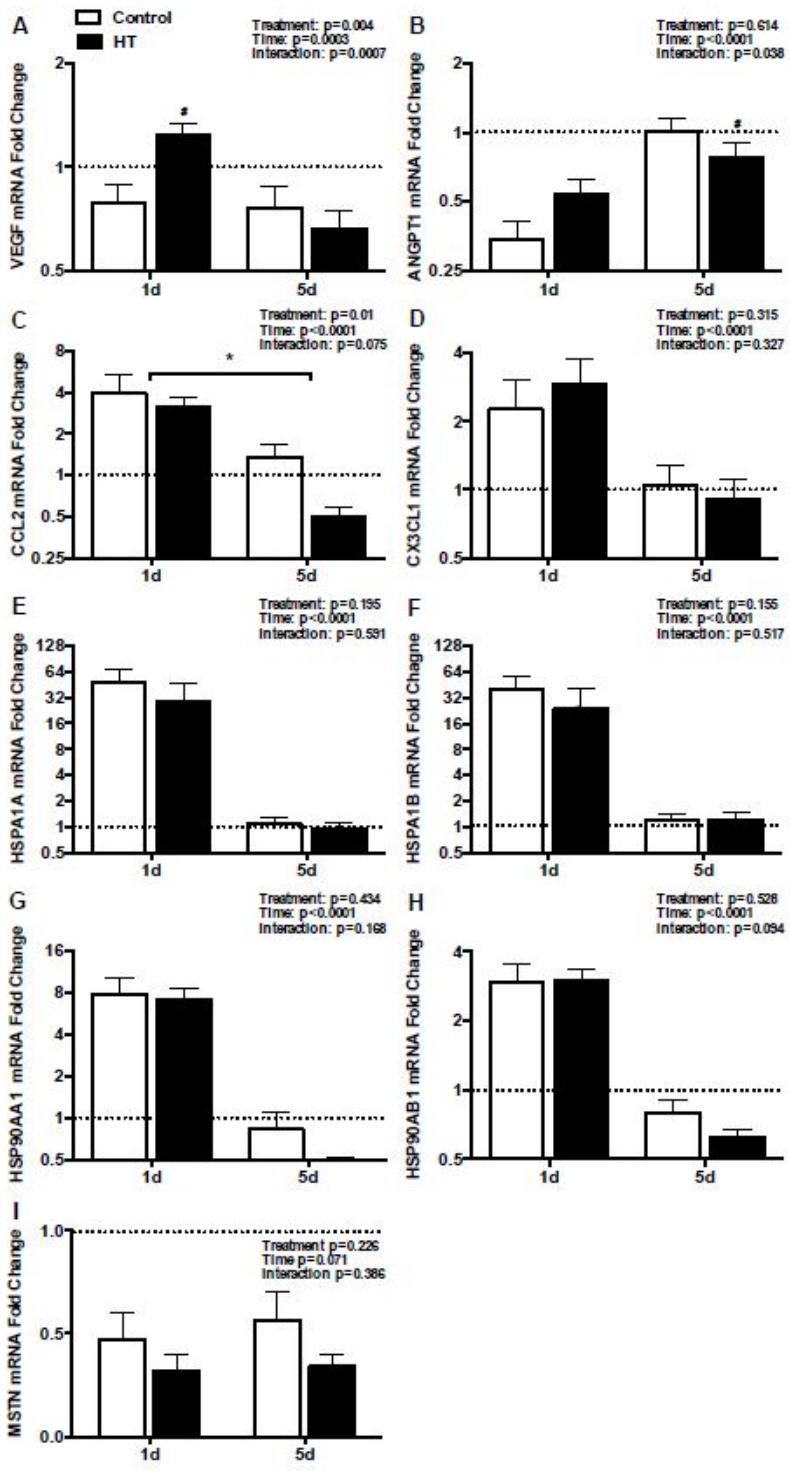


Figure 3.6: Fold changes in skeletal muscle mRNA expression relative to the baseline sample of select angiogenic, myogenic, inflammatory and stress management factors. Biopsy samples were obtained 2 weeks prior to and 1 and 5 days following the eccentric exercise bout. The baseline sample was assigned a value of 1 and is represented by the dashed line. Data were analyzed using a two-way repeated measures ANOVA. Values are means ± SE. * Main effect for treatment ($p=0.01$). $^{\#}$ $p<0.05$ vs. Control.

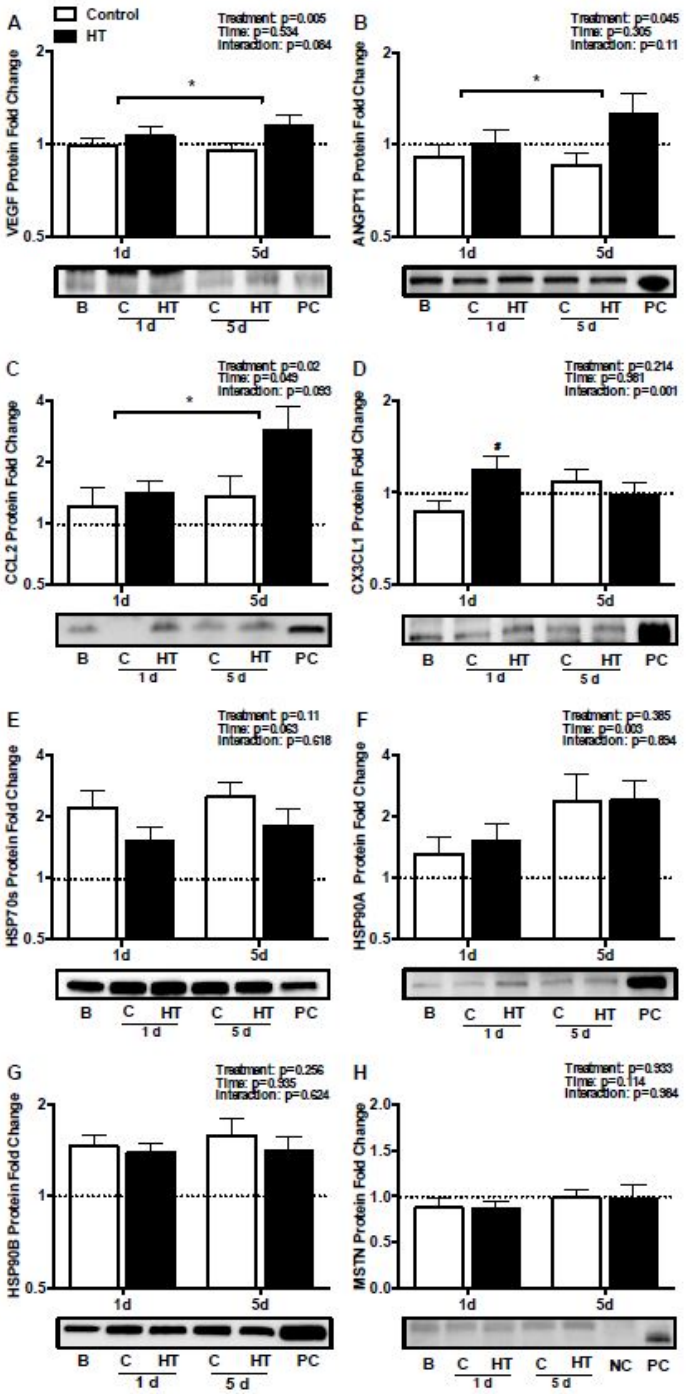
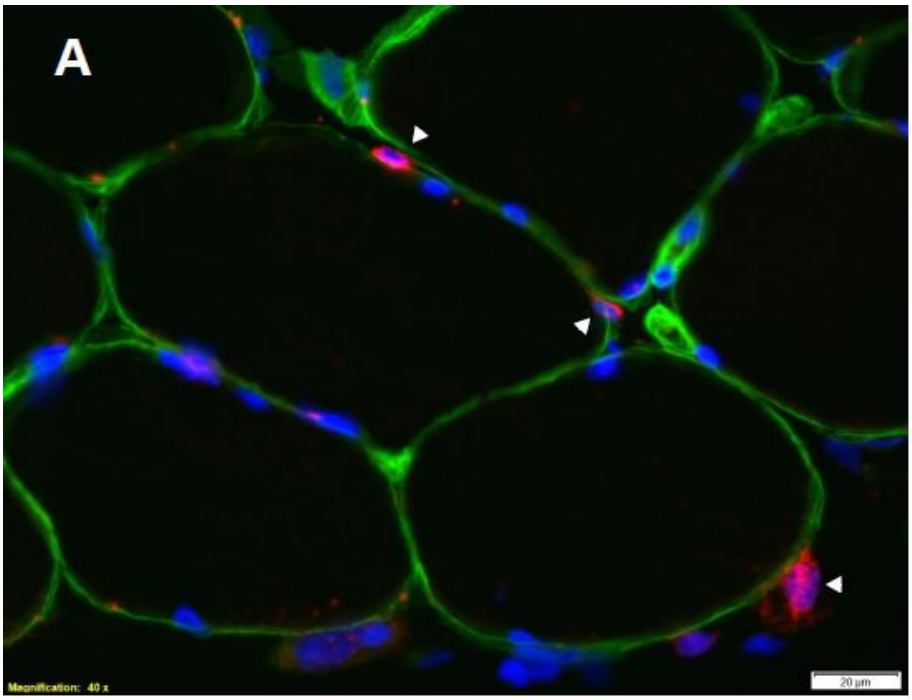


Figure 3.7: Fold changes in skeletal muscle protein expression relative to the baseline sample of select angiogenic, myogenic, inflammatory and stress management proteins. Biopsy samples were obtained 2 weeks prior to and 1 and 5 days following the eccentric exercise bout. The baseline sample was assigned a value of 1 and is represented by the dashed line. Representative western blots are shown below each panel. Data were analyzed using a two-way repeated measures ANOVA. Values are means ± SE. * Main effect for treatment ($p<0.05$). $^{\#}$ $p<0.05$ vs. Control. Abbreviations: B, baseline; C, control; HT, heat therapy; PC, positive control; NC, negative control.



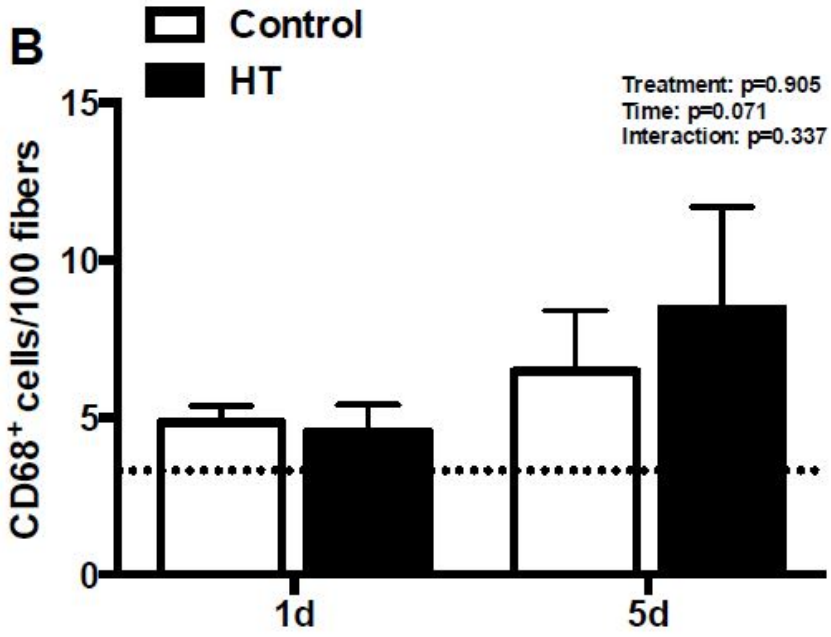


Figure 3.8: (**A**) Representative skeletal muscle cross sections displaying immunoreactivity for DAPI (blue), CD68 (red) and laminin (green). A predominant portion of $CD68^+$ immune-reactive macrophages were located between fibers (Panel A, arrowheads). Scale bar, 20 µm. **(B)** Number of $CD68^+$ cells per 100 fibers at 1 and 5 days following the eccentric exercise bout in the thigh treated with the control intervention (open bars) and the thigh treated with HT (solid bars). The baseline content of $CD68^+$ cells is represented by the dashed line (3.25 ± 0.35 cells per 100 fibers). A two-way repeated measures ANOVA was employed to compare the changes in the number of $CD68^+$ cells across the time course and between thighs. Values are means ± SE.

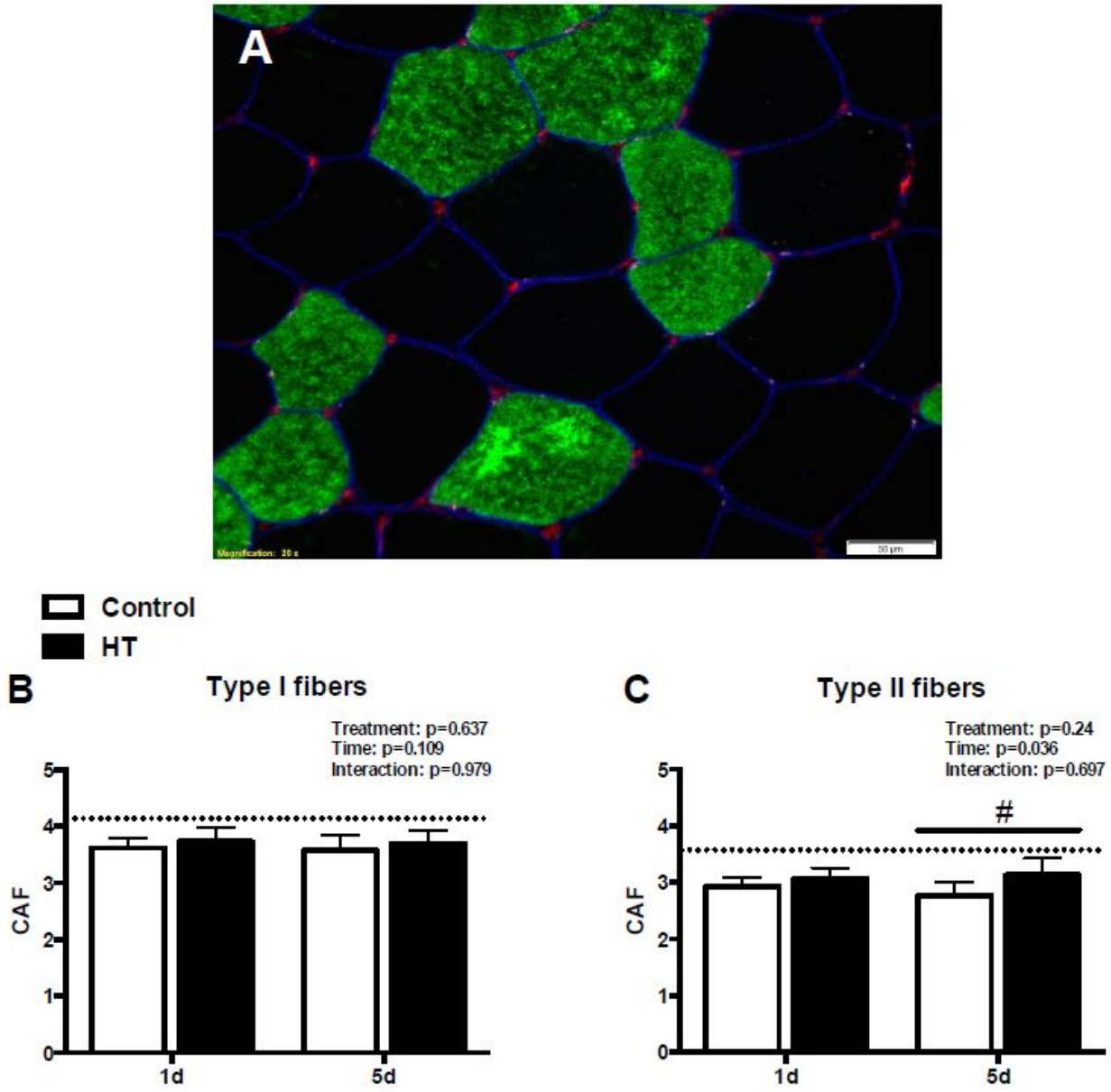


Figure 3.9: (**A)** Representative skeletal muscle cross section displaying immunoreactivity for dystrophin (blue), CD31 (red) and MHC-I (green). **(B-C)** The number of capillaries around each fiber (CAF) for type I (B) and type II fibers (C). The baseline CAF is represented by the dashed line (Type I: 4.19 ± 0.18 and Type II: 3.57 ± 0.24 capillaries around a fiber). A two-way repeated measures ANOVA was employed to compare the changes in CAF across the time course and between thighs. Values are means ± SD. # $p<0.05$ vs. Baseline.

CHAPTER 4. IMPACT OF REPEATED LOCAL HEAT THERAPY ON SKELETAL MUSCLE STRUCTURE AND FUNCTION IN HUMANS

Kyoungrae Kim[1], Blake A. Reid[1], Caitlin A. Casey[1], Brooke E. Bender[1], Bohyun Ro[1], Qifan Song[2], Adam J. Trewin[3], Aaron C. Petersen[3], Shihuan Kuang[4], Timothy P. Gavin[1], Bruno T. Roseguini[1]

[1]Department of Health and Kinesiology, Purdue University, West Lafayette, IN, USA

[2]Department of Statistics, Purdue University, West Lafayette, IN, USA

[3]Institute for Health and Sport, Victoria University, Melbourne, Victoria, Australia

[4]Department of Animal Sciences, Purdue University, West Lafayette, IN, USA

Running Title: Skeletal muscle adaptations to repeated local heat stress

Correspondence:

Bruno T. Roseguini, Ph.D.

Department of Health and Kinesiology, Purdue University

800 W. Stadium Avenue, West Lafayette, IN, 47907

E-mail: brosegui@purdue.edu

Phone: 765-496-2612; Fax: 765-496-1239

Abstract

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±1.4 years, BMI 24.9±0.9 kg/m^2) had one randomly selected thigh treated with HT (garment perfused with water at ~52ºC) for 8 consecutive weeks (90 min, 5 days/week) while the opposite thigh served as a control. Biopsies were obtained from the vastus lateralis muscle before and after 4 and 8 weeks 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: 4.1±3.7 Nm vs. HT: 9±4.6 Nm) and 8 (Control: 1.7±2.7 Nm vs. HT: 7.7±2.9 Nm). Exposure to HT had no impact on capillarization indices in type 1 fibers. Conversely, capillarization around type 2 fibers was higher in the thigh treated with HT as compared to the control thigh ($p<0.05$). The content of eNOS was ~18% and 35% higher in the thigh exposed to HT at 4 and 8 weeks, 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 CSA and mitochondrial content. These results indicate that exposure to local HT for 8 weeks promotes a pro-angiogenic environment and enhances muscle strength but does not affect mitochondrial content in humans.

Key words: heat therapy, skeletal muscle

New & noteworthy

We demonstrate that repeated application of heat therapy to the thigh using a garment perfused with warm water enhances the strength of knee extensors and impacts 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.

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 (3-5, 15) as well as in elderly patients with chronic heart failure (23, 27) and other cardiovascular diseases (16, 26, 28). 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 (21). The salutary effects of HT are thought to stem in part from beneficial changes in the cardiovascular system, including improved endothelial function, reduced arterial stiffness, and blood pressure (13). However, it is increasingly evident that HT also elicits positive changes in skeletal muscle structure and function. Treatment with whole-body HT for 6 weeks increased skeletal muscle capillary density and endothelial cell-specific endothelial nitric oxide synthase (eNOS) content in young individuals (15). Moreover, as few as eleven days of daily passive HT improves skeletal muscle contractility, as evidenced by an increase in evoked peak twitch amplitude and maximal voluntary torque production (24).

Although whole-body HT modalities have received the greatest attention, emerging evidence indicates that local HT may also promote skeletal muscle remodeling in humans. Passive heating of a small area or body segment typically induces minimal or no change in core body temperature and tends to be better tolerated than strenuous whole-body HT strategies such as hot water immersion and sauna (20). Goto and co-workers first reported that repeated local thigh heating increased isometric force production of the knee extensors in humans (9). Hafen and co-workers reported that short-term heat treatment promotes mitochondrial adaptations (11) and attenuates immobilization-induced atrophy in human skeletal muscle (10). We previously demonstrated that a single session of local thigh heating evokes increased skeletal muscle gene expression associated with vascular growth, including vascular endothelial growth factor (VEGF) (20). 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 impact of 8 weeks of exposure to local HT (5 days/week) on muscle strength, myofiber morphology and 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 ~52ºC 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 (20); and 2) five daily 90-min sessions of local HT hastens functional recovery following eccentric exercise-induced muscle damage (18). Based upon 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

Subjects

Twelve healthy young adults volunteered to participate in this study (mean ± SE: 23.6±1.4 y, 172.9±2.5 cm, 74.5±3.0 kg). Participants were asked to fill out a health and medical history questionnaire prior to enrollment. Exclusion criteria were: pregnancy, obesity (body mass index (BMI) > 30 kg/m^2), hypertension (resting systolic/diastolic blood pressure > 140/90 mm Hg), smoking, intake of medications and vitamin supplements, and history of deep vein thrombosis. Subjects that participated in any kind of supervised physical activity or engaged in physical activity more than 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

Subjects initially visited the laboratory on four separate occasions over a 2-3 week period. On visits 1 and 2, subjects 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 detailed below. These initial testing sessions were separated by a minimum of 48 hours. At least one week after visit 3, resting muscle biopsies were collected from the vastus lateralis of the left and right legs of each subject (18, 20). The 8-week intervention protocol commenced at least three days after the muscle biopsy procedures. Using a within-subject design,

the legs of participants were assigned in a counterbalanced fashion to receive HT or a control regimen. Subjects were asked to report to the laboratory 5 days per week for a total of 40 sessions. The length of the intervention (8 weeks) was based on the reports by Brunt and co-workers that 8 weeks of whole-body HT improves conduit vessel and cutaneous microvascular function (3, 4). Muscle strength and fatigability were reassessed after 4 and 8 weeks of treatment. These experimental sessions took place approximately 24 hours after the previous HT session. At least 48 hours 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 hours before undergoing muscle biopsies and to eat a light meal prior to the other experimental visits. Participants were instructed to abstain from vigorous physical activity in the 24 hours preceding each test and to avoid caffeine consumption on the day of testing. Subjects were asked to maintain their normal dietary and exercise behavior throughout the study. At the end of each week, subjects 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, Canada). This garment was customized with an extensive network of medical-grade polyvinyl chloride tubing that covered the thighs and buttocks (18, 20). In the thigh assigned to receive HT, water at ~52ºC was perfused through the garment for 90 min with a goal to increase leg skin temperature to ~39.5–40°C (18, 20). Previous studies that employed a similar approach revealed that this regimen causes muscle temperature to increase from a baseline of ~33-34ºC to approximately 37ºC (6, 12).

Assessment of muscle strength and fatigability

Knee extensor strength and fatigue resistance were assessed using an isokinetic dynamometer (Humac NORM, Computer Sports Medicine, Inc., Stoughton, MA, USA) as described previously (18). Participants were familiarized with the testing procedures twice before the baseline assessment. Subjects 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 180º/s, a set of three maximal contractions at an angular velocity of 180º/s, and a set of 40 consecutive maximal contractions at 180º/s.

On 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 between participants. Participants were asked to complete 3 maximal consecutive contractions at 180º/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 approximately 3 min and were then asked to perform a bout consisting of 40 consecutive maximal contractions at 180º/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.

Muscle sampling

Muscle biopsies were obtained from the vastus lateralis under local anaesthesia (Lidocaine hydrochloride, Hospira, Lake Forest, IL) using a 5-mm Bergstrom biopsy needle (Pelomi Medical, Albruslund, Denmark). The biopsy specimens were promptly weighed, cleared from visible fat and connective tissue, and divided into three sections. Approximately 40 mg sections were mounted in transverse orientation in a disposable base mold using 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 -80ºC until citrate synthase and Western blot analysis.

Immunohistochemistry

Transverse serial sections (10µm) of muscle were cut using a Leica CM1850 cryostat (Leica, Wetzlar, Germany) at -23°C, mounted on frosted microscope slides (Thermo Scientific, NH, USA), air-dried for 0.5-1 hours 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. Following 2 x 3 min washes with 1x 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 using primary antibodies against the basal lamina and myosin heavy chain (MHC) isoform proteins. Sections were incubated for 3 hours 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 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 hour at room temperature: Alexa Fluor 488 goat anti-rabbit IgG (A11008, 1:1000), Alexa Fluor 488 goat anti-mouse IgG2b (A21141, 1:1000), Alexa Fluor 568 goat anti-mouse IgG1 (A21124, 1:1000), and Alexa Fluor 350 goat anti-mouse IgM (A31552, 1:1000). All secondary antibodies were obtained from Thermo Fisher Scientific. Following 4 x 5 min washes, slides were briefly dried and mounted using fluorescent mounting medium (Dako, CA, USA) and the edges were sealed with nail polish (Sally Hansen Hard as Nails, NY, USA).

Identification of fiber type-specific capillaries was performed in neighboring sections using 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, DSHB). After 2 x 5 min washes with 1x PBS, sections were stained with appropriate secondary antibodies (Alexa 350 goat anti-rabbit IgG, A11609, 1:500; Alexa 488 goat anti-rabbit IgG, A11008, 1:1000; Alexa 488 goat anti-mouse IgG 2b, A21141, 1:1000; and Alexa 568 goat anti-mouse IgG1, A21124, 1:1000, Thermo Fisher Scientific), diluted in 1x 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 using an Olympus BX53 fluorescence microscope equipped with an Olympus DP72 digital camera and cellSens Dimension software. The entire specimen cross section was initially selected using the stage navigator. The multi-channel image was then acquired and two images from each channel were merged using Image J software (National Institutes of Health, USA). Histological analysis was not performed in 1 out of 72 samples due to insufficient muscle yield.

Analysis of immunofluorescence images

Analyses of immunofluorescence images were carried out using Adobe Photoshop CC 2015. Fiber type distributions were determined from counts of an average of 612 ± 70 muscle fibers (range 221–1260 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 using the following indices: (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 the C/Fi ratio divided by the fiber perimeter of a given fiber (14).

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, 1 mM EDTA (RIPA Lysis Buffer, EMD Milipore) with freshly added protease inhibitor cocktail (P8340, Sigma-Aldrich) and phosphatase inhibitors (50 mM NaF and 0.2mM Na_3VO_4) at a 1:15 dilution of wet muscle weight using a bead mill homogenizer (BEAD RUPTOR12, 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, USA). All samples were subsequently diluted with homogenization buffer (1.5 µg/µL) and subsequently mixed with either reducing sample buffer (4x Laemmli sample buffer with 10% 2-Mercaptoethanol) or non-reducing sample buffer (4x Laemmli sample buffer). Afterwards, samples were heated to 95ºC for 5 min (except for mitochondrial OXPHOS protein blots), divided into small aliquots, and stored at -80ºC.

Western blot analysis

For the analysis of HSP90A, HSP90B, VEGF, ANGPT1, p-eNOSser^{1177}, eNOS, and OXPHOS, 20 µg of protein were separated by SDS-PAGE on precast Stain Free 4-15 % gels (Bio-Rad, CA, USA) and transferred to polyvinylidene fluoride (PVDF) membranes using the Trans-

Blot® Turbo transfer system (Bio-Rad, CA, USA). Membranes were subsequently blocked with 5 % non-fat milk in 1x TBST (1% tween 20) solution for 1 h at room temperature (~23°C) and incubated for 3-4 hours at room temperature with primary antibodies diluted in blocking buffer. The membranes were washed with 1x TBST at room temperature for 3 x 10 min, incubated with horseradish peroxidase-conjugated secondary antibodies diluted in 1x TBST for 1 h at room temperature and were then washed with 1x TBST at least 3 x 10 min before being exposed to an enhanced chemiluminescent solution (Clarity Western ECL, Bio-Rad, USA) for 5 min. Membranes were visualized using a densitometer (ChemiDoc Touch Imaging System, Bio-Rad, USA), and band densities were determined using image-analysis software (Image Lab V6.0.1, Bio-Rad, USA). PageRuler Prestained Protein Ladder (Thermo Fisher, USA) was used as a molecular weight marker. Control for equal loading was performed using the 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, HSPA1A was performed as described previously (8). Details of the primary antibodies are provided in Table 1. Recombinant proteins were used to confirm antibody specificity.

Citrate synthase activity

The maximal enzyme activity of citrate synthase (CS) was determined using 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, pathlength 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% w/v 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 $\varepsilon = 14150\ M^{-1}.cm^{-1}$ at 412 nm) every 15 s over a 3-min period at 25ºC then enzyme activity was expressed as nanomoles per minute per milligram of protein.

Statistical analysis

All statistical analyses were conducted using SAS (Version 9.4; SAS Institute) with results expressed as means ± SE. The Kolmogorov-Smirnov test was used to assess the distribution of the data. Data exhibiting skewed distribution were log-transformed before statistical analysis. A two-way 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 39.7±0.1ºC, while in the control leg the average temperature was 32.4±0.3ºC (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 180º/s improved by 6% at week 4 and by 5% on week 8 (baseline: 140±11 Nm, 4 weeks: 149±14 Nm, 8 weeks:148±13 Nm) (Figure 2). Conversely, in the control thigh, peak torque increased by 2% and 1% at weeks 4 and 8, respectively (baseline: 142±12 N, 4 weeks: 146±12 N, 8 weeks: 144±12 N). 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 180º/s, was not altered after exposure to either HT (baseline: 4434±355 J, 4 weeks: 4404±378 J, 8 weeks: 4449±369 J) or the control treatment (baseline: 4309±323 J, 4 weeks: 4400±371 J, 8 weeks: 4321±332 J) (Figure 2).

Fiber type distribution and morphology

Muscle fiber cross-sectional area (CSA), perimeter, SF as well as fiber type distribution are shown on Table 2. There were no treatment, time or treatment x time effects for the changes in fiber cross-sectional area in both fiber types. Fiber type distribution was also not significantly impacted by HT.

Capillarization

The number of capillary contacts in type 1 fibers declined throughout the study in both the control thigh (baseline: 4.9±0.3, 4 weeks: 4.6±0.2, 8 weeks: 4.8±0.2) and in the thigh treated with HT (baseline: 5.1±0.2, 4 weeks: 4.7±0.2, 8 weeks: 4.7±0.2) (Figure 3). Exposure to HT also had no significant impact on other capillarization indices in type 1 fibers (Figure 3). Conversely, while the number of capillary contacts around type 2 fibers declined by nearly 10% in the control thigh (baseline: 4.6±0.1, 4 weeks: 4.1±0.1, 8 weeks: 4.2±0.1), exposure to HT prevented a temporal reduction in this parameter (baseline: 4.2±0.1, 4 weeks: 4.2±0.1, 8 weeks: 4.2±0.2). A significant treatment effect was observed for the changes in capillary contacts ($p=0.016$), the capillary-to-fiber ratio on an individual fiber basis ($p=0.007$) and the capillary-to-fiber perimeter exchange index ($p<0.001$) in type 2 fibers (Figure 3).

Mitochondrial content

Figure 4 and 5 display the changes in maximal citrate synthase activity and the content of OXPHOS protein complexes, respectively. There were no treatment, time, or treatment x time interaction for levels of the mitochondrial OXPHOS proteins measured.

Expression of angiogenic factors and heat shock proteins

A main effect of treatment was observed for the changes in skeletal muscle eNOS content ($p=0.003$), while eNOS ^{ser1177} phosphorylation ($p=0.389$) and eNOS phosphorylation normalized to eNOS content ($p=0.201$) were not altered by the intervention (Figure 6). The protein content of members of the small heat shock protein (HSP20) family, alpha B-crystallin (HSPB5) (main effect of treatment, $p=0.007$) and heat shock protein family B member 1 (HSPB1) (main effect of treatment, $p=0.009$), were also significantly higher in the thigh treated with HT (Figure 6). No treatment effect was observed for the changes in VEGF, ANGPT1, HSPA1A and the HSP90 family members (Figure 6).

Discussion

The primary findings of this study were that repeated local thigh heating for 8 weeks elicited an increase in eNOS content and averted a temporal decline in skeletal muscle capillarization indices when compared to a control intervention. Conversely, HT had no impact on

skeletal muscle mitochondrial content. Confirming earlier observations that exposure to local and whole-body heat stress improves skeletal muscle contractile function (9, 24), we also report that 8 weeks of local HT enhanced the strength of the knee extensors. Combined, these findings indicate that a simple and well-tolerated HT modality significantly impacts 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 (20). Thus, participants were required to spend 90 min daily (5 days/week) 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 due to time constraints. Although we did not directly measure physical activity patterns, analysis of weekly reports by the participants revealed that 7 individuals had marked decrements in exercise time throughout the study, while 3 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 cross-sectional area (Table 2) and capillarization (Figure 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) impairs glucose metabolism, including insulin sensitivity (19) and lowers myofibrillar protein synthesis rates (25) in healthy young adults. More severe forms of muscle disuse, such as 2 weeks of single leg limb immobilization, lead to reduced leg lean mass and muscle capillarization in old and young men (30). Of note, exposure to HT has been shown to attenuate the manifestations of skeletal muscle disuse in animals (37, 58) as well as in humans (10). Our findings that daily local HT prevented the decline and/or enhanced indices of capillarization (Figure 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.

Impact of HT on muscle capillarization

The ability of heat stress to promote a pro-angiogenic milieu in skeletal muscle and a consequent increase in capillarization was first documented by Akasaki and co-workers in a model of peripheral arterial insufficiency (1). These authors showed that mice treated with far-infrared dry sauna daily for 5 weeks had greater capillary density and eNOS expression in the ischemic muscle. Of note, chronic treatment with NOS inhibitor N(ω)-nitro-L-arginine methyl ester (L-NAME) abolished the changes in capillarization as well as the recovery in blood flow (1). Recently, these earlier observations in ischemic mouse skeletal muscle were extended to humans. Hesketh and co-workers reported that 6 weeks of whole-body passive HT increased capillary density by 21% and endothelial-specific eNOS content by 8% in the vastus lateralis muscle of sedentary young individuals (15). Combined, these studies provide compelling evidence implicating nitric oxide (NO) as a critical mediator of heat-induced skeletal muscle angiogenesis.

Based upon these earlier reports, we examined the impact of local HT on the content of eNOS and muscle capillarization. In accordance with the previous findings from whole body heating (15), we report that eNOS content was 18% and 35% higher in the thigh exposed to HT as compared to the control thigh at 4 and 8 weeks, respectively (Figure 6). Changes in eNOS were accompanied by significant differences in capillarization between HT and control in type 2, but not type 1 fibers (Figure 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, 15). Studies in animals (2) as well in humans (12) have documented a modest increase in muscle blood flow during exposure to local heat stress. It is possible to speculate that type 2 fibers experienced a greater relative increase in blood flow (and wall-shear stress) during HT compared to type 1 fibers. Alternatively, it is possible that the effects of HT were mostly evident in type 2 fibers because capillarization around these fibers was more severely impacted by reduced physical activity levels (Figure 3). Of note, Hesketh and co-workers did not observe differences between fiber types in the magnitude of the increase in capillarization following 6 weeks of whole-body HT (15).

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 following a single session (20) as well as 5 days of repeated exposure to HT in injured muscle (18). It might be possible that the time lag between the

last HT session and biopsy sampling (72 hours) precluded us from detecting a treatment effect. Another possible interpretation is 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 (Figure 6). 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 (7, 17). Additional studies are warranted to define the role these molecular chaperones exert on heat-induced skeletal muscle angiogenesis.

Heat stress and mitochondrial biogenesis

The finding that heat stress induces mitochondrial biogenesis in C2C12 myotubes (22) has led to several investigations asking if 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 (29). More recently, local heating of the vastus lateralis for 6 consecutive days (2 h daily) increased mitochondrial respiratory capacity and mitochondrial content (11). In contrast, we did not observe a significant impact 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 co-workers that repeated whole-body HT had no impact on skeletal muscle mitochondrial density despite marked effects on exercise capacity and capillarization (15). The inconsistent effect of HT on mitochondrial content and function 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 shortwave diathermy (11), which produces rapid and marked deep tissue heating, may be more effective at producing mitochondrial adaptations than superficial heat modalities as employed in the current report.

HT and skeletal muscle strength

Given that local heat treatment of the thigh for 8 h/day for 10 weeks improved maximal isometric force in young individuals (9), we questioned if 90 min of thigh heating would

significantly enhance knee extensor strength. In agreement with the findings of Goto and co-workers (9), maximal isokinetic torque increased to a greater extent in the thigh exposed to HT as compared to the control thigh with just 90 min of treatment (Figure 2). This improvement in force after treatment with local HT occurred despite no significant differences in fiber cross-sectional area between treatments (Table 2), indicating that adaptations other than changes in fiber size explain the observed improvements in force generating capacity. Of note, as little as 11 days of whole-body heat stress increased peak twitch amplitude and torque production of the plantar flexors in humans (24). 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 (24). Further research is needed to explore the cellular basis of enhanced force-generating capacity of muscles exposed to repeated heat stress.

Clinical implications

The key advantage of water-circulating garments, blankets and pads over other methods of passive heat stress is that these devices are easy to use and accessible. Indeed, water-circulating garments perfused with warm water 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 or other HT modalities. Our findings that local HT enhances muscle strength and impacts muscle capillarization indicate that this method may be a feasible tool to treat conditions associated with capillary rarefaction and muscle weakness.

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Conflicts of interest:

None

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Table 4.1. Antibodies used for Western blotting

Antigen	Primary antibody	Secondary antibody	Recombinant protein
Heat shock protein 90A	Bio-Rad (AHP2473)	Bio-Rad (anti-rabbit STAR121P)	Bio-Rad (VMA00081KT)
~ 90 kDa			
Reducing	1:2000	1:10000	
Heat shock protein 90B	Bio-Rad (VMA00082)	Bio-Rad (anti-mouse STAR207P)	Bio-Rad (VMA00082KT)
~ 90 kDa			
Reducing	1:2000	1:10000	
VEGF165	R&D system (AF-293-NA)	R&D system (anti-goat HAF017)	R&D system (293-VE-010)
~ 39 kDa			
Non-reducing	1:200	1:10000	
Angiopoietin 1	R&D system (AF923)	R&D system (anti-goat HAF017)	R&D system (923-AN-025)
~70 kDa			
Reducing	1:250	1:10000	
p-eNOS^{ser1177}	abcam (215717)	Bio-Rad (anti-rabbit STAR121P)	abcam (198066)
~140 kDa			
Non-reducing	1:1000	1:10000	
eNOS	abcam (199956)	Bio-Rad (anti-rabbit STAR121P)	abcam (198066)
~140 kDa			
Non-reducing	1:1000	1:10000	
OXPHOS	abcam (110413)	Bio-Rad (anti-mouse STAR207P)	abcam (110413)
~20 to ~55 kDa			
Reducing, non-heated	1:1000	1:10000	
HSPB1	Enzo (ADI-SPA-800)	Perkin Elmer (anti-mouse NEF822001EA)	NA
~ 27 kDa			
Reducing	1:5000	1:50000	
HSPB5	Enzo (ADI-SPA-222)	CST (anti-mouse 7076)	NA
~ 22 kDa			
Reducing	1:1000	1:5000	
HSPA1A	Enzo (ADI-SPA-810)	Perkin Elmer (anti mouse NEF822001EA)	NA
~ 70 kDa			
Reducing	1:8000	1:50000	

Table 4.2. Muscle fiber morphological measurements

	Control			Heat therapy		
	Week 0	**Week 4**	**Week 8**	**Week 0**	**Week 4**	**Week 8**
Type I CSA, μm^2	6013.3±328.1	-20.3±347.0	-533.5±344.5	6394.2±464.5	-431.9±517.9	-315.5±645.0
Type II CSA, μm^2	7186.8±481.6	-443.9±473.0	-1192.3±565.4	6960.6±348.8	-488.6±645.6	-564.3±558.3
Type I perimeter, µm	326.2±8.1	7.3±9.8	-11.7±9.7	339.2±12.3	-18.4±11.3	-6.2±19.5
Type II perimeter, µm	367.4±17.6	-14.2±17.3	-35.7±19.6	359.0±10.3	-23.7±13.5	-17.8±18.1
Type I SF	2.53±0.05	-0.06±0.09	0.07±0.05	2.62±0.02	0.02±0.04	-0.10±0.06
Type II SF	2.58±0.04	-0.03±0.09	0.05±0.05	2.59±0.05	0.05±0.07	-0.06±0.08
Type I (%)	38.4±1.8	-2.01±2.6	-1.45±1.5	42.5±4.0	-3.82±2.7	-2.73±3.5
Type II (%)	61.6±1.8	2.01±2.6	1.45±1.5	57.5±4.0	3.82±2.7	2.73±3.5

Values are means ± SE; Week 0, baseline values prior to treatments; Week 4 and week 8, changes from baseline value following 4 and 8 weeks of heat therapy or control intervention

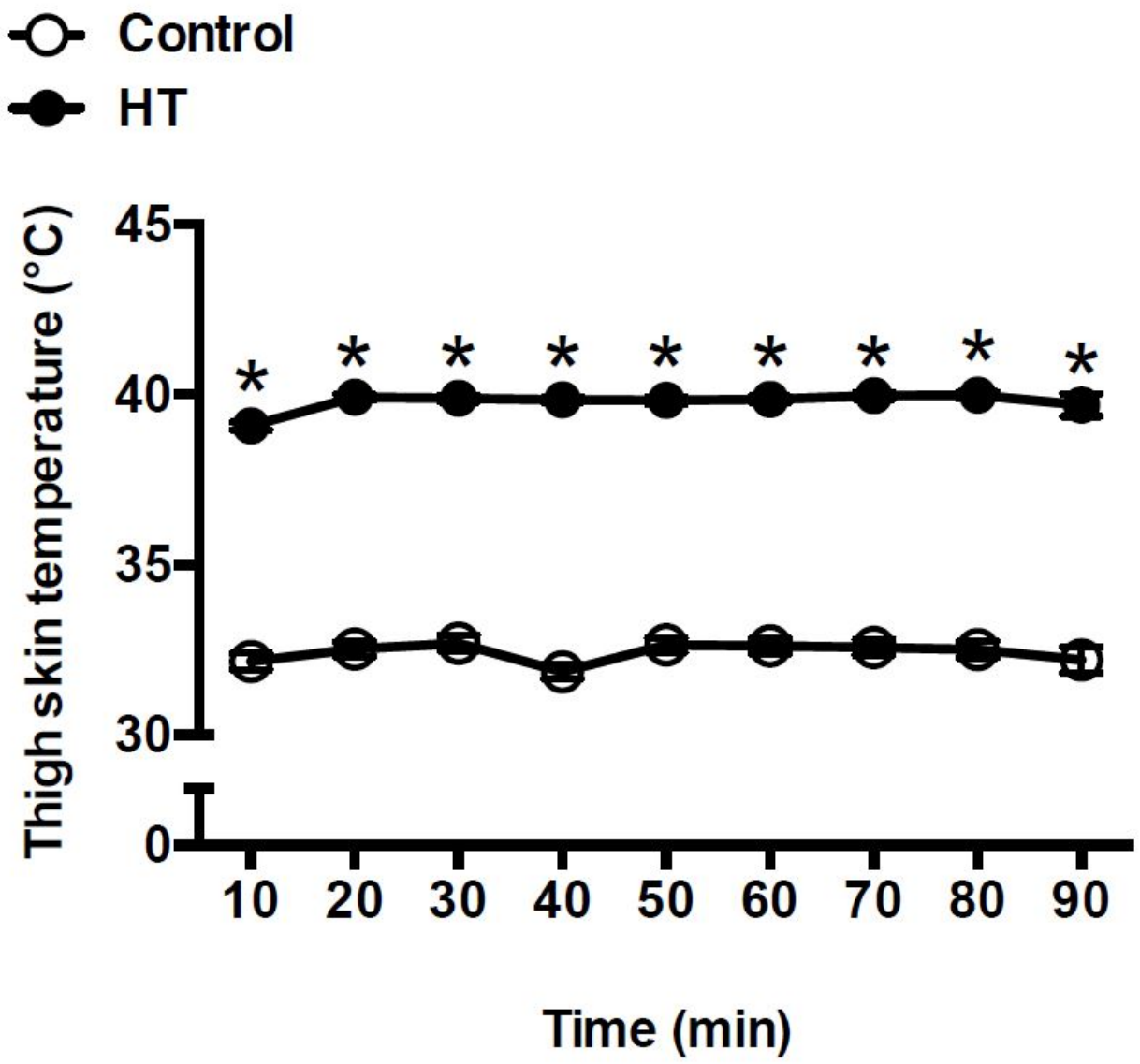

Figure 4.1: Thigh skin temperature during exposure to 90 min of heat therapy (HT, closed circles) or the control intervention (open circles). Data were analyzed with a 2-way repeated-measures ANOVA. Values are means ± SE. *$p < 0.05$ vs. Control.

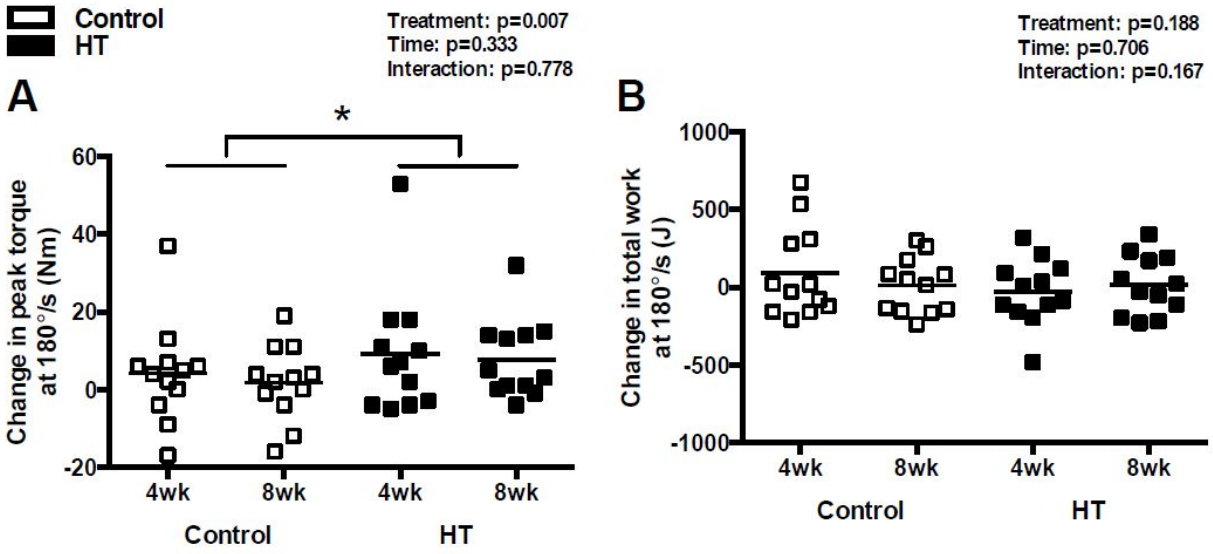

Figure 4.2: Individual and group mean changes from baseline in muscle strength (A) and fatigue resistance (B) following 4 and 8 weeks of heat therapy (HT, closed squares) or the control intervention (open squares). Data were analyzed with a 2-way repeated-measures ANOVA. *main effect of treatment ($p < 0.05$).

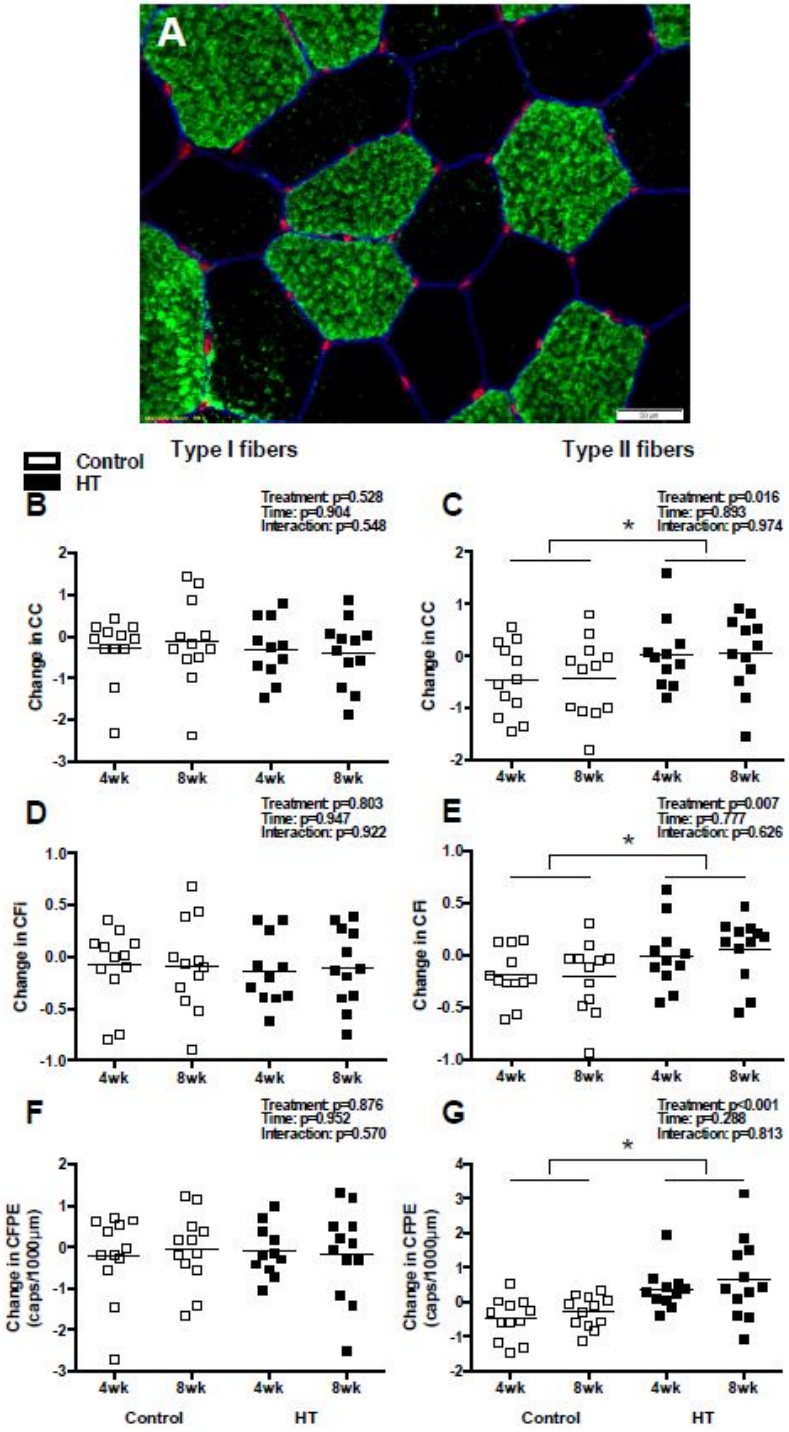

Figure 4.3: A: Representative skeletal muscle cross section displaying immunoreactivity for dystrophin (blue), CD31 (red), and myosin heavy chain type I (green). B and C: Changes from baseline in the number of capillary contacts (CC) for type I (B) and type II (C) fibers. D and E: Changes from baseline in the 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 ($p < 0.05$).

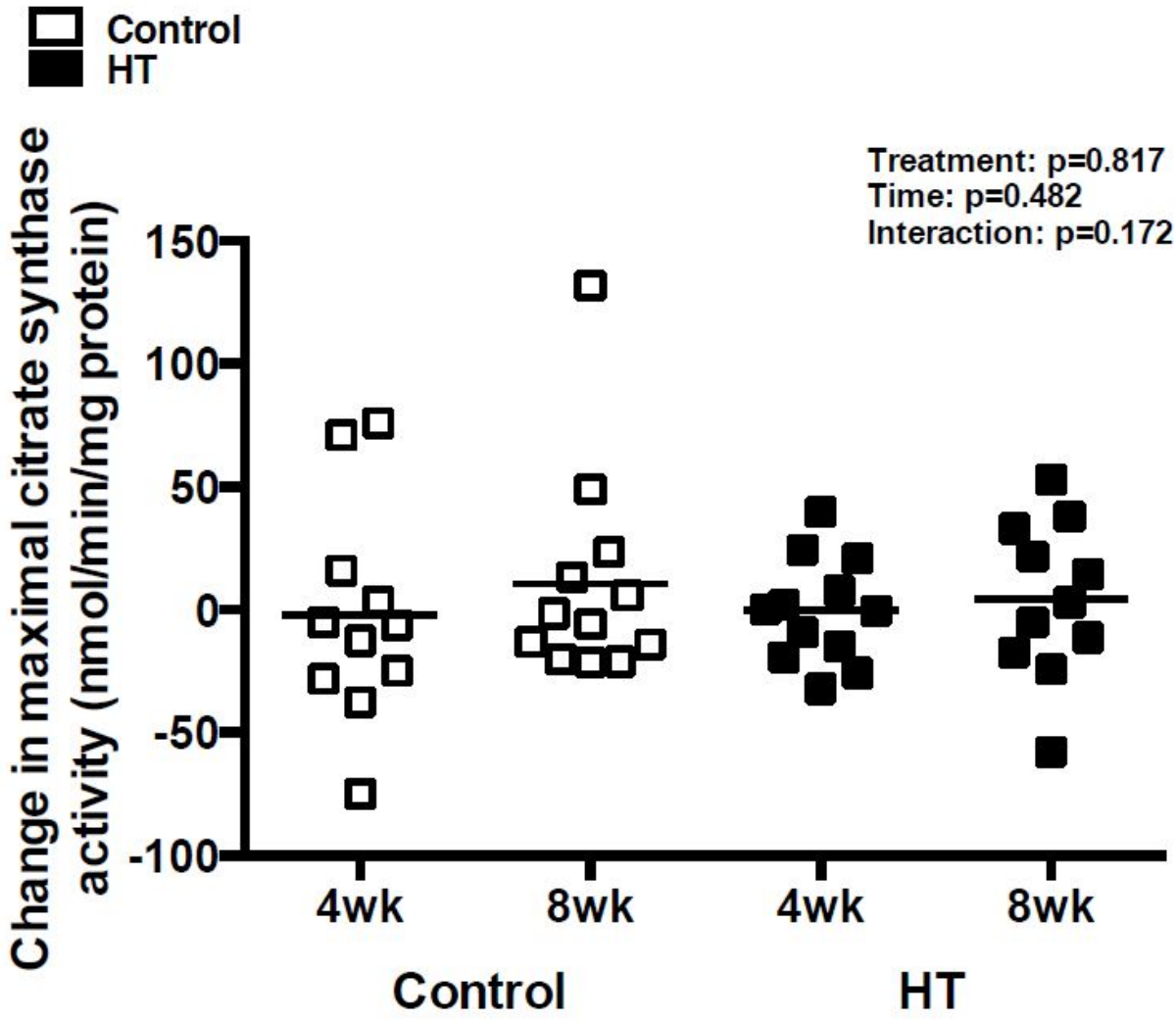

Figure 4.4: A: Individual and group mean changes from baseline in maximal citrate synthase activity following 4 and 8 weeks of heat therapy (HT, closed squares) or the control intervention (open squares).

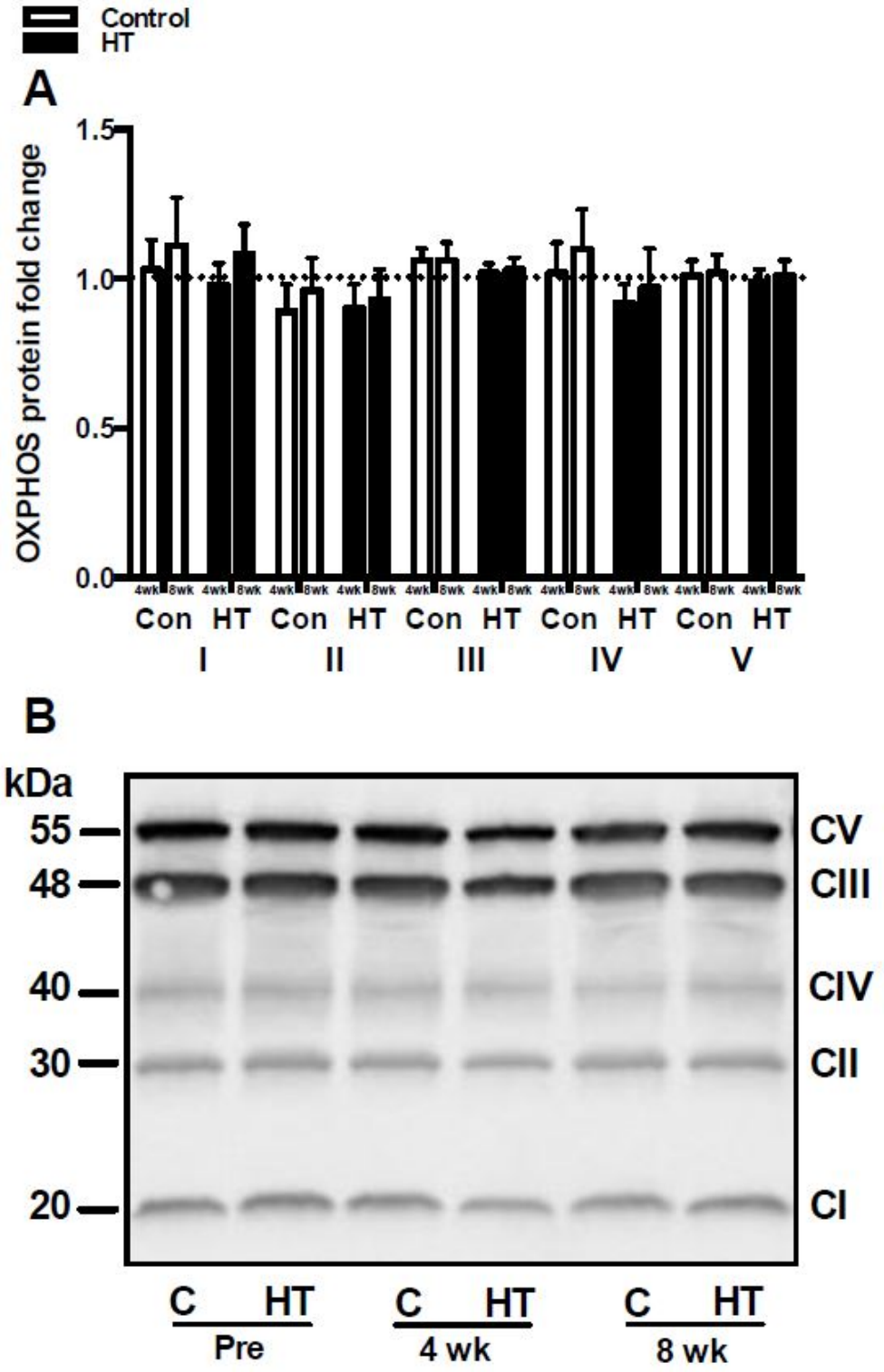

Figure 4.5: A: Fold changes in protein expression relative to the baseline sample of mitochondrial respiratory chain complexes (I-V). B: Representative blot displaying the five mitochondrial oxidative phosphorylation complexes. The baseline sample was assigned a value of 1 and is presented as a dashed line. Data were analyzed with a 2-way repeated-measures ANOVA. Values are means ± SE.

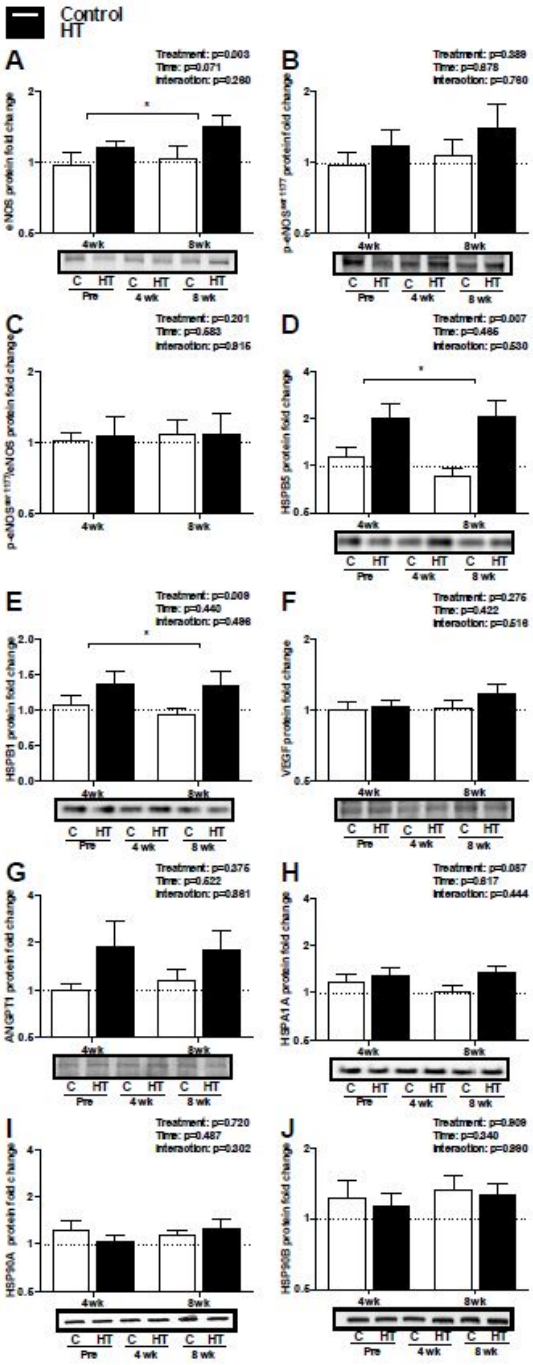

Figure 4.6: Fold changes in skeletal muscle protein expression relative to the baseline sample of select stress management and angiogenic proteins. A: Endothelial nitric oxide synthase (eNOS). B: Phosphorylated endothelial nitric oxide synthase at Ser1177 (p-eNOS ^{ser1177}). C: The ratio of p-eNOS^{ser1177} to eNOS. D: Alpha B-crystallin protein (HSPB5). E: Heat shock protein family B member 1 (HSPB1). F: Vascular endothelial growth factor (VEGF). G: Angiopoietin 1 (ANPTT1). H: Heat shock protein 72-kDa (HSPA1A). I: Heat shock protein 90-kDa alpha class A member 1 (HSP90A). J: Heat shock protein 90-kDa alpha class B member 1 (HSP90B). The baseline sample was assigned a value of 1 and is represented by the dashed line. Data were analyzed with a 2-way repeated-measures ANOVA. Values are means ± SE. *main effect of treatment ($p < 0.05$).

CHAPTER 5. HEAT THERAPY IMPROVES SOLEUS MUSCLE FORCE IN A MODEL OF ISCHEMIA-INDUCED MUSCLE DAMAGE

Kyoungrae Kim[1], Blake A. Reid[1], Bohyun Ro[2], Caitlin A. Casey[1], Qifan Song[3], Shihuan Kuang[4], Bruno T. Roseguini[1]

[1]Department of Health and Kinesiology, Purdue University, West Lafayette, IN, USA

[2]Department of Physical Education, Dong-A University, Busan, Korea

[3]Department of Statistics, Purdue University, West Lafayette, IN, USA

[4]Department of Animal Sciences, Purdue University, West Lafayette, IN, USA

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Correspondence:

Bruno T. Roseguini, Ph.D.

Department of Health and Kinesiology, Purdue University

800 W. Stadium Avenue, West Lafayette, IN, 47907

E-mail: brosegui@purdue.edu

Phone: 765-496-2612; Fax: 765-496-1239

Abstract

Leg muscle ischemia in patients with peripheral artery disease (PAD) leads to alterations in skeletal muscle morphology and reduced leg strength. We tested the hypothesis that exposure to heat therapy (HT) would improve skeletal muscle function in a mouse model of ischemia-induced muscle damage. Male 42-week-old C57Bl/6 mice underwent ligation of the femoral artery and were randomly assigned to receive HT (immersion in a water bath at 37°C, 39°C, or 41°C for 30 min) or a control intervention for 3 weeks. At the end of the treatment, the animals were anesthetized and the soleus and extensor digitorium longus (EDL) muscles were harvested for the assessment of contractile function and examination of muscle morphology. A subset of animals was used to examine the impact of a single HT session on the expression of genes involved in myogenesis and the regulation of muscle mass. Relative soleus muscle mass was significantly higher in animals exposed to HT at 39ºC as compared to the control group (Control: 0.36±0.01mg/g vs. 39ºC: 0.40±0.01mg/g, $p=0.024$). Maximal absolute force of the soleus was also significantly higher in animals treated with HT at 37ºC and 39ºC (Control: 274.7±6.6mN, 37ºC: 300.1±7.7mN, 39ºC: 299.5±10mN, $p<0.05$). In the soleus, but not the EDL muscle, a single session of HT enhanced the mRNA expression of myogenic factors as well as of both positive and negative regulators of muscle mass. These findings suggest that the beneficial effects of HT are muscle-specific and dependent on the treatment temperature in a model of PAD.

Key words: heat therapy, skeletal muscle, hindlimb ischemia

New & noteworthy

This is the first study to comprehensively examine the impact of temperature and muscle fiber type composition on the adaptations to repeated heat stress in a model of ischemia-induced muscle damage. Exposure to heat therapy (HT) at 37ºC and 39ºC, but not at 41ºC, improved force development of the isolated soleus muscle. These results suggest that HT may be a practical therapeutic tool to restore muscle mass and strength in patients with peripheral artery disease.

Introduction

Patients with peripheral artery disease (PAD) have severe exercise intolerance and faster rates of functional decline and mobility loss when compared to age-matched counterparts (30-34). The genesis of functional impairment in these patients has been primarily ascribed to the reduction in leg blood flow capacity caused by atherosclerotic narrowing of arteries supplying the lower extremities (6), but accumulating evidence indicates that alterations in skeletal muscle structure and function also play an important role (1, 48, 49). Histopathological analysis of biopsy samples from the calf muscles of patients with PAD reveal a plethora of abnormalities, including myofiber degeneration and necrosis, fatty infiltration and fibrosis (10, 11, 19, 20, 24, 51). Lower extremity ischemia is also associated with smaller calf muscle area and a consequent impairment in functional capacity (29, 32). Several studies documented a substantial reduction in the strength of calf muscles in patients with PAD (4, 35, 51, 55). Of particular importance, poor leg strength is associated with faster functional decline (12) and higher mortality (35) in patients with PAD. Thus, therapeutic strategies that combat the causes of PAD myopathy and improve leg strength have the potential to improve mobility and increase survival of these patients.

Preclinical rodent models of hindlimb ischemia have been extensively used to explore the genetic basis and mechanisms underlying the PAD myopathy (25, 26, 47, 50, 56, 57) as well as to test novel interventions (52-54). One commonly used strategy to induce acute limb ischemia and a consequent injury of the calf muscles is the surgical occlusion of femoral artery in mice (46). The ischemic insult leads to myofiber necrosis, mitochondrial functional abnormalities and a prolonged deficit in the muscle force-generating capacity (46). Some therapies, such as antioxidant treatment (52) and a mitochondria-targeting peptide (53) have shown to improve muscle regeneration and muscle function in these animal models, but an urgent need remains for the development of new, non-pharmacological and widely accessible therapies to treat the manifestations of PAD myopathy.

One emerging therapeutic strategy that might be useful to reverse the abnormalities associated with skeletal muscle ischemic injury is exposure to heat therapy (HT). A growing number of studies in animal models of muscle injury induced by crushing or injection of toxins indicate that exposure to HT markedly improves skeletal muscle regeneration (18, 42, 61, 62). Treatment with HT has also been shown to attenuate skeletal muscle atrophy during immobilization and unloading (37, 58), rescue denervation-induced atrophy and mitochondrial

clearance (39, 63) and increase mitochondrial content and oxidative capacity (64). In addition, we recently reported that treatment with HT immediately after and for 4 consecutive days following exercise-induced muscle damage resulted in improved fatigue resistance of the knee extensors in humans (16).

The exact mechanisms by which HT accelerates muscle regeneration following injury and attenuates muscle atrophy during unloading have not been fully elucidated, but there is evidence that exposure to heat stress: 1) promotes the expression of myogenic regulatory factors (9), 2) facilitates the activation and proliferation of satellite cells (42, 43, 62), 3) stimulates intracellular signaling pathways involved in muscle protein synthesis (15, 39, 68), 4) suppresses proteolytic systems (63, 69), 5) inhibits injury-related collagen infiltration (61, 62), 6) enhances the expression of several members of the heat shock protein family (HSPs) (44, 45), molecular chaperones that play vital roles in a number of physiological processes, including skeletal muscle regeneration (60). In addition, we recently reported that HT promotes the expression of regulatory factors involved in muscle repair, including vascular endothelial growth factor (VEGF) and angiopoietin 1 (ANGPT1) (16, 22).

The primary aim of the present study was to test the hypothesis that 3 weeks of daily exposure to hindlimb immersion in a warm water bath would improve regeneration and enhance muscle force in a model of ischemia-induced muscle damage. To test this hypothesis, we randomly divided animals in groups that received HT at 37°C, 39°C, or 41°C for 30 min daily. At the end of the intervention, we assessed the contractile function and morphology of the slow-twitch soleus and the fast-twitch extensor digitorium longus (EDL) muscles. To gain insights into the potential molecular mechanisms underlying the therapeutic effects of HT, we examined the impact of a single 30-min HT session on the expression of select genes involved in myogenesis and the regulation of muscle mass. Based upon the aforementioned studies in models of muscle injury and atrophy, we hypothesized that HT would elicit an increase in the mRNA expression of HSPs, pro-myogenic factors and mediators of protein synthesis and hypertrophy.

Methods

Animals

Male C57BL/6J mice (42 weeks of age) were obtained from the Jackson Laboratory (Bar Harbor, ME) and were housed in groups of three to five in a cage (18 × 29 cm and 12.5 cm height)

in a climate-controlled room (22 ±1ºC, 60% humidity, 12:12-h light dark photoperiod). Water and regular chow (Teklad global 18% protein rodent diet, Envigo Bioproducts Inc, Madison, WI) were given ad libitum. All animal procedures were approved by the Institutional Animal Care and Use Committee at Purdue University (#1604001389) and conformed to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health.

Experimental protocol

Mice were allowed to acclimate to the vivarium for a minimum of 3 days before undergoing study procedures. Animals either underwent bilateral distal femoral artery ligation or a sham operation as described in detail below. After 2 weeks of recovery from the surgical procedure, mice with femoral artery ligation were randomly allocated to a control group or to one of three HT groups: 37ºC, 39ºC and 41ºC. In the first series of experiments, the control intervention and HT were applied for 30 minutes, 6 times per week over 3 consecutive weeks. Sham-operated animals were also exposed to the control regimen. The treatment duration and frequency were chosen based on the report by Nonaka and co-workers that a similar regimen attenuates muscle atrophy induced by streptozotocin in rats (38). Forty-eight hours following the last treatment session, the animals were anesthetized via an isoflurane and oxygen mixture and right hindlimb was excised. The soleus and EDL muscles were isolated for the assessment of contractile muscle function and histological analysis. These two muscles were chosen because, in addition to the contrasting fiber type composition, their geometry and size are ideal for isolated muscle preparations (36).

A second series of experiments was conducted to examine the acute impact of HT on skeletal muscle gene expression in mice that underwent femoral artery ligation. After recovering from the bilateral ligation procedure for 2 weeks, animals were exposed to a single 30-min session of HT or the control regimen. Immediately after the end of the intervention, the animals were anesthetized via an isoflurane and oxygen mixture and the soleus and EDL muscles from both legs were harvested, cleared from visible fat and connective tissue, blotted dry, frozen in liquid nitrogen and stored at -80ºC until RNA extraction. The timing of tissue collection (i.e. immediately after the end of the HT or control session), was based on the report of Tamura and co-workers that a single exposure to whole-body heat stress for 30 min in mice induces a prompt activation in the soleus and plantaris muscles of the mammalian target of rapamycin (mTOR) complex 1 (mTORC1) pathway, which is critically involved in the regulation of muscle protein synthesis (64).

Femoral artery ligation.

Bilateral ligation of the femoral artery was performed as described previously (52). Mice were anesthetized via an isoflurane and oxygen mixture and an incision was aseptically made from the origin of the saphenous artery to the groin. The femoral artery was carefully dissected away from the vein and nerve and ligated with a 6-0 nonabsorbable silk suture (Sofsilk™ black braided silk, Covidien) at a point distal to the superficial epigastric artery and proximal to the femoral artery's trifurcation into the saphenous, popliteal, and geniculate arteries (2). In the sham-operated, group, the femoral artery was dissected free but not ligated. The incision was then closed with a 5-0 sterile absorbable suture (Vicryl undyed, Ethicon) and the animal was given carprofen (5 mg/kg) subcutaneously for postoperative analgesia.

Heat therapy

Heat therapy was applied by placing the animal in a flat bottom restrainer (model 541-RR; Plas-labs Inc., Lansing, MI) and immersing the lower half of the body in a glass container filled with water (Figure 1). The animals were habituated to the restraining device for 6 days prior to the onset of the intervention. A maximum of 4 restraining devices were placed in the container at a given time. Water temperature was continuously monitored throughout the intervention. Animals assigned to the control regimen were also restrained, but were placed in an empty container. Rectal temperature was measured before and during exposure to a single session of HT or the control intervention in a subset of animals (n=3/group) using a thermistor probe (RET-3) connected to computer controlled multi-channel thermometer (Iso-Thermex, Columbus Instruments, Columbus, Ohio, USA).

Assessment of contractile function

Under isoflurane anesthesia, the right hindlimb was excised and immediately placed in a bicarbonate-buffered solution (in mmol/l: 137 NaCl, 5 KCl, 1 $MgSO_4$, 1 NaH_2PO_4, 24 $NaHCO_3$, and 2 $CaCl_2$) equilibrated with 95% O_2-5% CO_2 (pH ~7.4) for dissection. The assessment of contractile function of the EDL muscle was performed first, followed by the soleus muscle. After surgical isolation, braided silk suture thread (4-0, Fine Science Tools, Foster City, CA) was tied around the proximal and distal muscle tendons. The muscles were then excised and mounted in an *in vitro* muscle test system (1200A Intact Muscle Test System, Aurora Scientific, ON, Canada)

continuously bubbled with carbogen (5% CO_2 in O_2) at room temperature. The stimulation protocol consisted of supramaximal electrical current delivered through platinum electrodes using a biphasic high-power stimulator (701C; Aurora Scientific). The muscles were positioned at the length that elicited the highest twitch force (optimal length, L_o) and the temperature of the organ bath was increased to 32°C. After 10 min of thermoequilibration, a force-frequency relationship was generated using select frequencies between 1 and 300 Hz for the EDL muscle (300-ms train) and 1 and 200 Hz for the soleus muscle (500-ms train). This was followed by a 5 min fatiguing protocol consisting of 500 ms volleys of 40 Hz stimulation applied once every 2 s. The muscle was then removed from the organ bath, trimmed of connective tissue, blotted dry, and weighed. Muscle cross sectional area (CSA) was determined by dividing the wet muscle mass by the product of L_0 and muscle-specific density (1.056 g/cm^3). Specific force (N/cm^2) was calculated by dividing the muscle force (N) by the CSA (cm^2). The maximum twitch response was analyzed for peak twitch tension, time to peak twitch tension and twitch half-relaxation time.

Histological analysis

After the conclusion of the contractile function assessment, soleus and EDL muscles were pinned to a Sylgard-coated petri dish containing 10% neutral buffered formalin (Sigma-Aldrich). After approximately 1 hour, the muscles were transferred to a small container with formalin and allowed to fix at room temperature for 72 hours prior to histological processing. The muscles were then sectioned at the mid-belly and embedded in paraffin. Serial transverse sections (4 μm) were cut on a Leica CM300 microtome and routinely stained with hematoxylin and eosin (H&E) for the enumeration of fibers with centralized nuclei, which represent regenerating myofibers. Masson's trichrome staining was used to assess the content of fibrillar collagen deposition within injured muscle. Slides were scanned and digitized at 20× resolution on an Aperio Scanner (Aperio ScanScope XT; Aperio Technologies). The number of fibers with centralized nuclei was manually counted and is expressed as percentage of all fibers. Masson's trichrome–stained slides were analyzed using ImageScope software (Aperio, Vista CA). The fibrotic area is expressed as a percentage of positive pixels to total pixels.

RNA extraction

Frozen muscles (~10 mg) were homogenized in 1 ml of TRIzol reagent (Invitrogen, Carlsbad, USA) using a bead mill homogenizer (BEAD RUPTOR12, Omni International). The RNA was separated into an aqueous phase using 0.2 ml of chloroform and precipitated from the aqueous phase using 0.50 ml of isopropanol. Extracted RNA was washed with 1 ml of 75% ethanol, dried, and then suspended in nuclease-free water. RNA concentration was determined using the Nanodrop 3000 Spectrophotometer (Thermo Fisher Scientific) as described previously (22). The quality and integrity (RIN of 9.0 ± 0.1) of extracted RNA (401.9 ± 19.5 ng/µl) was evaluated using an RNA 6000 Nano LabChip kit on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). One microgram of total RNA was reverse transcribed into cDNA according to the manufacturers' directions (iScript, BioRad, Hercules, CA).

Skeletal muscle gene expression

Duplicates of cDNA samples were amplified on a CFX96 real-time PCR detection system (Bio-Rad), using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). The oligonucleotide primers are shown in Table 1. 18S rRNA was used as the internal reference gene and proved stable across all interventions. The comparative Ct method was used to calculate the changes in gene expression of each target mRNA relative to the control group (23).

Statistical analysis:

All statistical analyses were conducted using SAS (Version 9.4; SAS Institute) with results expressed as means ± SE. The Kolmogorov-Smirnov test was used to assess the distribution of the data. Data exhibiting skewed distribution were log-transformed before statistical analysis. Comparisons between sham-operated animals and the control group that underwent bilateral ligation of the femoral artery were performed using Student's t-test. Body mass changes throughout the intervention and the force-frequency relationships were compared between ligated groups using a two-way repeated measures ANOVA. All other data were analyzed by one-way ANOVA. Post-hoc analysis (Dunnett's) was performed when appropriate. For all analyses, $P < 0.05$ was considered statistically significant.

Results

Rectal temperature

Figure 1 (panel B) depicts the changes in rectal temperature during exposure to a single 30-min session of HT or the control regimen. Prior to the intervention, rectal temperature was similar between groups (Control: 35.3±0.4ºC, 37ºC HT: 35.5±0.2ºC, 39ºC HT: 35.4±0.6ºC, 41ºC HT: 35.5±0.4ºC). Upon exposure to HT, rectal temperature rose promptly and achieved steady-state levels within ~10 min. The average rectal temperature in the last 5 min of the intervention was 36.6 ±0.1ºC, 38.1±0.4ºC and 39.7±0.3ºC in the HT groups exposed to water at 37ºC, 39ºC and 41ºC, respectively. In the control group, rectal temperature remained stable throughout the 30-min intervention.

Body and muscle masses

There were no significant group differences in body mass before and throughout the 3-wk intervention (treatment main effect, p=0.68). After the first week of treatment, body mass decreased significantly (~4%) on all experimental groups and remained stable in the subsequent weeks (Figure 2, panel A). There were no differences in EDL and soleus muscle masses between sham-operated animals and the control group with femoral artery occlusion (Table 2). Absolute mass of the soleus muscle was approximately 7% greater in animals exposed to HT at 37ºC and 39ºC as compared to the control group (Table 2), albeit this difference did not reach statistical significance (treatment main effect, p=0.21). Relative soleus muscle mass (muscle mass-body mass ratio) was significantly greater (11%) in animals treated with HT at 39ºC when compared to mice exposed to the control intervention (p=0.02, Figure 2, panel B). Absolute (Table 2) and relative (Figure 2, panel C) EDL muscle masses did not differ between ligated groups.

Contractile function

The twitch contraction characteristics of the soleus and EDL muscles of animals subjected to bilateral ligation of the femoral artery and treated with the control regimen differed from those of the sham-operated group (Table 2). In the soleus muscle, both time-to-peak twitch tension and half-relaxation time were significantly slower ($p<0.05$) in the muscles of animals with femoral artery occlusion. Contrarily, in the EDL muscle, both parameters were significantly faster ($p<0.05$) in the muscles of ligated animals (Table 2). Maximal absolute and specific forces of the soleus and

EDL muscles are shown on Figure 3. Soleus specific isometric tetanic force was significantly lower in the animals with femoral artery occlusion when compared to sham-operated animals ($p=0.002$) (Figure 3, panel C). No group differences were detected for EDL absolute and specific forces (Figure 3, panel B and D).

Exposure to HT did not alter twitch contraction parameters (peak twitch tension, time to peak twitch tension and half-relaxation time) (Table 2). The force-frequency relationships of the soleus and EDL muscles are displayed on Figures 4 and 5. Soleus absolute isometric tetanic force in animals treated with HT at 37ºC and 39ºC was significantly higher than the control group between stimulation frequencies of 120 and 200 Hz (treatment x frequency interaction, $p<0.001$) (Figure 4, panel A). No group differences were detected when force was normalized to the estimated cross-sectional area (Figure 4, panel C). There was no significant effect of HT treatment on the absolute and specific force-frequency relationships in the EDL muscle (Figure 5, panels A and C). During repeated tetanic contractions at 40Hz, the decrease in relative force (as a percentage of initial force) was similar between groups in both the soleus and EDL muscles (data not shown).

Skeletal muscle morphology

The percentage of fibers with central nucleation, a marker of muscle regeneration, was similar between groups in both the soleus and EDL muscles (Figure 6, panels C and D). HT also had no impact on the collagen content measured as a fraction of total muscle area in both the soleus and EDL muscles (Figure 7, panels C and D).

Heat shock protein expression

Figure 8 displays the changes in the mRNA expression of several members of the HSP family following a single 30-min session of HT or the control intervention. In the soleus muscle, a temperature-dependent increase in the expression of Hspa1b (Hsp70) (treatment main effect, $p<0.001$) and Hsp90aa1 (Hsp90a) (treatment main effect, $p<0.001$) was observed. Conversely, the expression of Hspd1 (Hsp60) was significantly increased relative to the control regimen only in the animals treated with HT at 37ºC ($p=0.039$). No changes were detected between groups in Hsp90ab1 (Hsp90b). Similar responses were detected in the EDL muscle, with the exception of Hsp60, which was not significantly different between groups.

Expression of pro-myogenic factors

Exposure to HT did not significantly impact the mRNA expression of Myog (myogenin) and Myod1 (MyoD) in either the soleus or the EDL muscle. When compared to the control group, Pax7 ($p=0.012$) and Vefga ($p=0.006$) mRNA expression in the soleus muscle was significantly increased in animals exposed to HT at 37ºC. No group differences were observed in the expression of these factors in the EDL muscle (Figure 9).

Expression of positive regulators of muscle growth

The expression of a few select genes involved in signaling pathways that mediate protein synthesis are shown on Figure 10. In the soleus, but not the EDL muscle, the mRNA expression of the serine-threonine protein kinases 1 and 2 (Akt1 and Akt2) was significantly higher in the group exposed to HT at 37ºC when compared to the control group (Akt1, $p=0.004$; Akt2, $p=0.0062$). A similar trend was observed in the 39ºC group for both Akt1 ($p=0.1$) and Akt2 ($p=0.06$). Conversely, HT had no impact on the expression of Igf1 and Rps6kb1 in either the soleus or the EDL muscle.

Expression of negative regulators of muscle growth

Figure 11 displays the changes in the mRNA expression of a few select regulators of muscle proteolysis, apoptosis and atrophy following a session of HT or the control intervention. The expression of Trim63 (MuRF1), Capn2 and Fbxo32 were significantly higher in the soleus muscle of animals exposed to HT at 37ºC when compared to the control group (Trim63, $p=0.025$; Capn2, $p=0.024$; Fbxo32, $p=0.014$). No significant group differences were observed in the expression of Mstn, Capn1 and Foxo3 in either the soleus or the EDL muscle.

Discussion

We investigated the impact of 3 weeks of daily treatment with HT on the morphology and contractile function of the soleus and the EDL muscle in a model of ischemia-induced muscle damage produced by femoral artery ligation in mice. The primary findings of these experiments were that the effects of HT depended on both treatment temperature and muscle fiber-type composition. Repeated immersion in water at 37ºC and 39ºC, but not at 41ºC, promoted an increase in force development in the isolated slow-twitch soleus muscle. Conversely, no detectable changes

were observed in the EDL muscle, indicating that fast-twitch muscles might be less prone or unresponsive to the beneficial effect of HT on contractile function in this animal model. Examination of the transcriptional changes that occur following a single session of HT also revealed a muscle-specific response. In the soleus, but not the EDL, exposure to HT evoked the activation of myogenic factors as well as of both positive and negative regulators of muscle mass. Taken together, these novel findings contribute to the growing body of literature that supports a beneficial impact of HT in conditions associated with impaired muscle function and atrophy.

Rodent models of peripheral arterial insufficiency induced by surgical occlusion of the femoral artery have been extensively employed not only to investigate the possible genetic causes but also to test potential therapies to reverse ischemia-induced skeletal muscle abnormalities (66). Clinically relevant models typically incorporate risk factors that are associated with the development and/or progression of vascular insufficiency, including aging (21). We chose to study adult (i.e. 10 month-old) rather than young C57BL/6J mice because aging is a major risk factor for increased ischemic tissue injury (3). It has been shown that 10 month-old mice display a diminished recovery of flow relative to 4 month-old animals following femoral artery ligation (65). We report that the ischemic insult induced by ligation of the femoral artery caused extensive muscle damage and a persistent deficit in the force generating capacity of the soleus muscle (Figure 3). In the group subjected to femoral artery ligation and assigned to the control intervention, soleus maximal absolute and specific forces were 8% and 14% lower, respectively, than in sham-operated mice. These findings are congruent with those of Hourdé and co-workers that maximal specific force of the medial gastrocnemius muscle was reduced by ~23% four weeks following surgical ligation of the femoral artery in mice (14). Of note, however, we observed that maximal force of the fast-twitch EDL muscle was comparable between ligated controls and sham-operated animals. These findings, alongside with the observation that the number of fibers with central nucleation were ~15% lower in the EDL relative to the soleus (Figures 6) indicate that the magnitude of the ischemic injury is reduced and/or the rate of recovery is accelerated in the fast-twitch EDL in this animal model.

To test the hypothesis that treatment with HT would promote recovery from ischemia-induced muscle damage and improve muscle function, we exposed mice to daily hindlimb immersion in warm water. We selected this strategy, as opposed to other HT modalities, because: 1) numerous reports indicate that repeated warm water immersion accelerates recovery following

muscle injury (42, 61) and attenuates muscle atrophy following denervation (39); 2) the translational potential of this regimen is high given that water immersion is widely accessible and practical for patients with restricted locomotion and multiple comorbidities, such as patients with symptomatic PAD.

A seminal finding of the present study was that repeated exposure to HT enhanced force development of the soleus muscle. Although it is firmly established that heat stress accelerates regeneration and promotes positive adaptations in several rodent models of skeletal muscle injury (18, 42, 61, 62), the impact of HT on contractile function of leg muscles remains largely unexplored. We demonstrated that maximal isometric tetanic force was nearly 10% higher in animals treated with HT at 37ºC and 39ºC as compared to the control group. Of note, specific muscle force was not affected by HT, which indicates that the increase in muscle strength can be largely ascribed to changes in muscle mass. Absolute and relative soleus muscle masses in the animals exposed to HT at 39ºC were ~7% and ~12% greater than the control group, respectively. These observations are in line with accumulating evidence that repeated exposure to heat stress enhances muscle mass in healthy skeletal muscle (39-41) and attenuates inactivity-induced reductions in limb muscle mass (37, 39, 58, 59, 63).

The increases in muscle mass induced by repeated heat stress appear to stem from the stimulation of protein synthesis and possibly the inhibition of pathways involved in protein degradation. Yoshihara et al. (68) and several others (39, 40, 64) documented that heat stress activates the Akt/mTOR signaling pathway, which is a key mediator of protein synthesis and hypertrophy in skeletal muscle. In animal models of disuse or denervation-induced atrophy, HT has also been shown to suppress apoptotic and/or proteolytic systems (39, 69). We examined the impact of a single session of HT on the mRNA expression of select factors involved in both hypertrophy and atrophy. Our findings reveal that, although HT up-regulated the expression of Akt1 and Akt2, members of the Akt/mTOR signaling pathway, it also evoked increased expression of negative regulators of muscle mass, including MuRF1, Capn2 and Fbxo32. In agreement with our observations, Guo and co-workers recently reported that in C2C12 cells, exposure to hyperthermia promoted the expression of Akt1 and Akt2 and a number of atrophy-related genes, including Capn2 and Fbxo32 (7). These findings indicate that the transcriptional response to HT is complex and that the net effect of the treatment on muscle mass likely reflects a fine balance between protein synthesis and degradation.

The salutary effects of HT on muscle mass and force development were not observed in the EDL muscle in the present study, indicating that the adaptations to repeated heat stress are muscle specific. Since contractile function of the EDL in mice subjected to femoral artery ligation was comparable to those of sham-operated animals, it is possible to speculate that the lack of response of this fast-twitch muscle to HT is due to a ceiling effect. This seems unlikely given the notion that HT has been shown to stimulate protein synthesis and elicit increases in muscle mass in healthy skeletal muscle in rodents (17, 39, 64). Alternatively, it is conceivable that the adaptations to HT depend on muscle fiber type composition. The findings of some (13, 17), but not all (64), previous studies support the notion that muscles composed predominantly of slow-twitch fibers appear to more responsive to HT than fast-twitch muscles. For example, Kobayashi and co-workers reported that wet and dry weights of the soleus, but not EDL muscle, were increased 7 days following a single exposure to heat stress for 60 min in male Wistar rats (17). It has been suggested that a differential activation of HSP in response to HT could possibly make slow muscles more responsive to stressful conditions than fast muscles (45). Oishi and co-workers demonstrated that the soleus muscle had a more rapid and broader HSP response than the fast-twitch plantaris muscle following a single session of heat stress in rats (45). Activation of HSPs, molecular chaperones that are vital for cellular stress management, is thought to contribute to increases in muscle mass and other benefits elicited by exposure to heat stress (40). In the present study, the increases in the mRNA expression of Hsp60, Hsp70, Hsp90a and Hsp90b following a single session of HT were comparable between the soleus and the EDL muscle, indicating that activation of these stress genes does not appear to explain the observed muscle-specific adaptations to HT.

One intriguing finding of the present study was that soleus force development was enhanced in the groups treated with HT at 37ºC and 39ºC, but no detectable changes were observed in the animals immersed in water at 41ºC. These findings contrast with data from prior studies in healthy male rats that indicated that stimulation of protein synthesis and the consequent changes in muscle mass following HT were progressively greater at increasing temperatures. Yoshihara and co-workers immersed the legs of rats in water at 37, 38, 39, 40 or 41°C for 30 min and observed a temperature-dependent, incremental increase in the phosphorylation of Akt and its downstream target, p70S6K (68). Goto and co-workers (5) reported that the magnitude of increase in soleus muscle mass-body mass ratio 7 days after a single session of HT was also dependent on treatment

temperature, with greater changes observed in animals exposed to an environmental chamber heated to 41ºC. It is worth highlighting that these studies examined the impact of a single HT treatment session and therefore offer limited insights into the effects of temperature on the outcomes of repeated, long-term exposure to heat stress. Our findings reveal, for the first time, that during chronic HT treatment, a temperature threshold exists beyond which the beneficial effects of HT are negated. Interestingly, evidence derived from cell culture studies indicates that prolonged exposure to heat stress at 41ºC impairs myogenic differentiation, while 39ºC facilitates myogenesis and enhances myotube diameter (7, 67).

In addition to stimulating protein synthesis, it has been proposed that HT accelerates recovery following injury by inhibiting fibrosis and promoting the activation and proliferation of satellite cells (42, 61). Oishi and co-workers were among the first to report that repeated heat stress induced a marked increase in the number of $Pax7^+$ satellite cells as well as myonuclear number following muscle injury induced by bupivacaine in rats (42). In a similar model of injury, Shibaguchi and co-workers observed that treatment with HT for 2 weeks not only facilitated the proliferation of $Pax7^+$ cells but also reduced collagen infiltration in skeletal muscle (61). Although we did detect a significant increase in Pax7 mRNA expression in the soleus muscle of animals treated with HT at 37ºC, no measurable changes were observed in the expression of myogenic factors myogenin and MyoD. Exposure to HT also had no impact on collagen content and the number of fibers with central nucleation, a marker of muscle regeneration. Nonetheless, it is important to highlight that HT was not started until 2 weeks after the ischemic insult in the present study. It remains to be determined whether similar effects are observed if HT is applied in the early, immediate phase of injury, when satellite and immune cells are recruited to mount the regenerative response.

Limitations

An important limitation of our study was that we only examined the immediate effects of HT on skeletal muscle gene expression. Since the temporal profile of expression differs between genes, a single time point offers a rather limited view of the transcriptional response profile to heat stress in skeletal muscle. Also, the time course and magnitude of changes in mRNA and protein are often discordant, indicating that the increases in mRNA alone cannot predict whether or to what extent the protein levels of a given factor will be altered by the intervention. Thus, additional

studies are warranted to comprehensively document the acute and long-term impact of HT on the protein levels of regulators of skeletal muscle mass.

Another limitation of the current study was that we did not assess the behavior of EDL and soleus muscle temperatures during exposure to the different HT regimens. Previous studies in rats documented the temporal changes in soleus muscle temperature during hindlimb immersion in a water bath at different temperatures (39, 68). These reports indicated that at the end of a single 30-min HT session, soleus muscle temperature was similar to the temperature of the water bath (39, 68). Of note, these aforementioned studies in rats were performed under anesthesia, which is known to significantly impact body temperature regulation in rodents. It remains to be determined if these findings hold true in unanesthetized animals and whether the EDL displays a similar rate of temperature change as the soleus muscle upon exposure to HT.

Clinical implications

Muscle atrophy and the loss in leg muscle strength appear to contribute significantly to the functional impairment in patients with PAD (28, 51). Accordingly, therapies that restore muscle mass and improve leg muscle strength have the potential of enhancing exercise tolerance in these patients. For example, supervised lower extremity resistance training has been shown to enhance leg strength and improve treadmill walking performance and quality of life in patients with PAD with and without intermittent claudication (27). Unfortunately, few patients with this condition have access or adhere to supervised exercise training regimens (8). Conversely, leg immersion in warm water is practical for patients with multiple comorbidities that cannot undergo standard exercise regimens and is amenable for combination with other established approaches employed for PAD treatment. Our findings of improved force development after exposure to HT lays the foundation for future studies to examine the long-term impact of repeated HT on leg strength and functional capacity in patients with PAD.

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Conflicts of interest:

None

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Table 5.1. List of primers

Gene name	Primer sequence (5'-3')	Gene name	Primer sequence (5'-3')
Hspd1	F:CACAGTCCTTCGCCAGATGAG R:CTACACCTTGAAGCATTAAGGCT	Igf1	F:GTGAGCCAAAGACACACCCA R:ACCTCTGATTTTCCGAGTTGC
Hspa1b	F:GAGATCGACTCTCTGTTCGAGG R:GCCCGTTGAAGAAGTCCTG	Akt1	F:ATGAACGACGTAGCCATTGTG R:TTGTAGCCAATAAAGGTGCCAT
Hsp90aa1	F:AATTGCCCAGTTAATGTCCTTGA R:CGTCCGATGAATTGGAGATGAG	Akt2	F:ACGTGGTGAATACATCAAGACC R:GCTACAGAGAAATTGTTCAGGGG
HSP90ab1	F:GTCCGCCGTGTGTTCATCAT R:GCACTTCTTGACGATGTTCTTGC	Trim63	F:GTGTGAGGTGCCTACTTGCTC R:GCTCAGTCTTCTGTCCTTGGA
Vegfa	F:GCACATAGAGAGAATGAGCTTCC R:CTCCGCTCTGAACAAGGCT	Fbxo32	F:CAGCTTCGTGAGCGACCTC R:GGCAGTCGAGAAGTCCAGTC
Angpt1	F:CACATAGGGTGCAGCAACCA R:CGTCGTGTTCTGGAAGAATGA	Mstn	F:AGTGGATCTAAATGAGGGCAGT R:GTTTCCAGGCGCAGCTTAC
Pax7	F:TCTCCAAGATTCTGTGCCGAT R:CGGGGTTCTCTCTCTTATACTCC	Capn1	F:ATGACAGAGGAGTTAATCACCCC R:GGCTATGAGAAACCGGAGGG
Myod1	F:CCACTCCGGGACATAGACTTG R:AAAAGCGCAGGTCTGGTGAG	Capn2	F:GGAGAGAGGCTGTACCTTCCT R:CCGAGGTGGATGTTGGTCTG
Myog	F:GAGACATCCCCCTATTTCTACCA R:GCTCAGTCCGCTCATAGCC	Foxo3	F:CTGGGGGAACCTGTCCTATG R:TCATTCTGAACGCGCATGAAG
Rps6kb1	F:AGACACAGCGTGCTTTTACTT R:GTGTGCGTGACTGTTCCATCA	Rn18s	F:AGTCCCTGCCCTTTGTACACA R:CGATCCGAGGGCCTCACTA

Table 5.2. Muscle mass and twitch contraction parameters of isolated soleus and EDL muscles from the sham-operated animals and ligated animals exposed to the control treatment or HT at 37ºC, 39ºC or 41ºC.

	Soleus					EDL				
	SO	Control	37°C	39°C	41°C	SO	Control	37°C	39°C	41°C
Muscle mass (mg)	10.6±0.5	11.9±0.4	12.7±0.3	12.8±0.4	11.9±0.4	13.6±0.6	14.1±0.4	14.9±0.4	14.7±0.4	13.9±0.4
Peak twitch tension (mN)	32.3±3.6	33.4±1.5	33.9±1.2	34.2±1.6	33.6±1.5	49.9±11.7	41.4±2.8	42.9±2.5	42.0±2.7	40.0±3.7
Time to peak twitch tension (ms)	22.1±0.7*	23.7±0.3	22.4±0.4	22.6±0.5	23.5±0.4	13.1±0.2*	12.1±0.3	12.3±0.2	12.0±0.3	12.4±0.3
Half-relaxation time (ms)	21.4±1.1*	24.2±0.8	22.3±0.8	22.8±1.3	23.9±0.6	13.0±0.4*	11.8±0.2	12.0±0.2	11.9±0.2	11.7±0.2

Comparisons between sham-operated animals (SO) and the control group that underwent bilateral ligation of the femoral artery were performed using Student's t-test. The groups subjected to femoral artery ligation were compared using a one-way ANOVA. Values are means ± SE. *$p<0.05$ vs. Control. N=8 in the SO group and 14-16 in the Control, 37ºC, 39 ºC and 41 ºC groups.

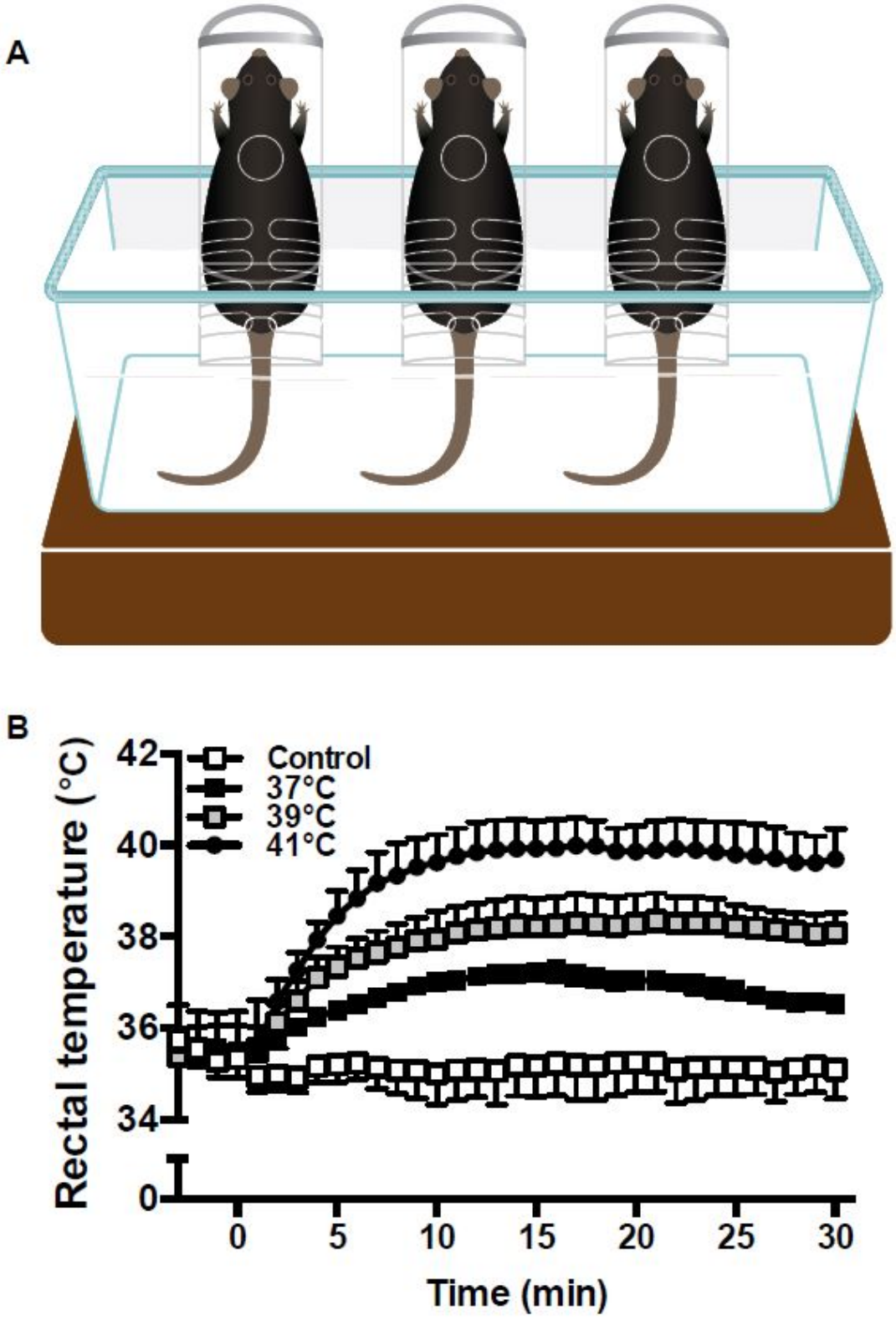

Figure 5.1: *Panel A*: Experimental set up for HT application. *Panel B*: Changes in rectal temperature before and during exposure to HT at 37ºC, 39ºC, 41ºC or the control intervention. Values are means ± SE. N=3 per group.

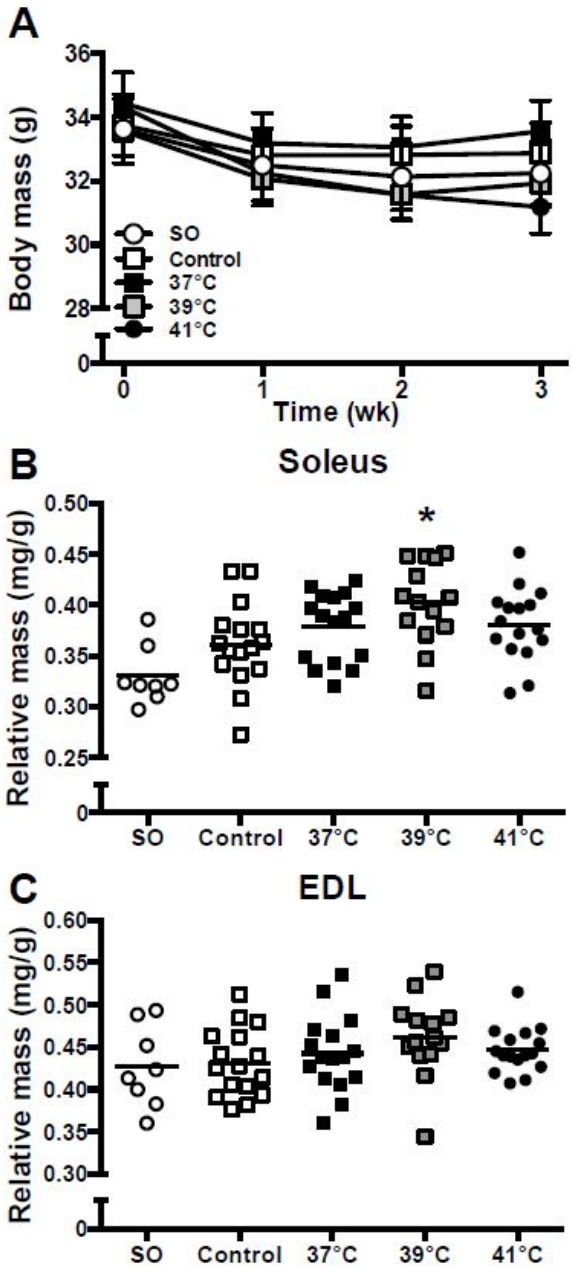

Figure 5.2: *Panel A*: Changes in body mass throughout the 3-wk intervention in the sham-operated group (SO) and in the groups subjected to femoral artery ligation and treated with the control regimen or HT at 37ºC, 39ºC or 41ºC. Data were analyzed using a two-way repeated measures ANOVA. *Panels B and C*: Individual and group mean relative soleus (*panel B*) and EDL (*panel C*) muscle masses. Comparisons between ligated groups were performed using a one-way ANOVA. Dunnett's post-hoc test was performed when appropriate. Values are means ± SE. * $p<0.05$ vs. Control. N=8 in the sham-operated group and 14-16 in the Control, 37ºC, 39ºC, and 41ºC groups.

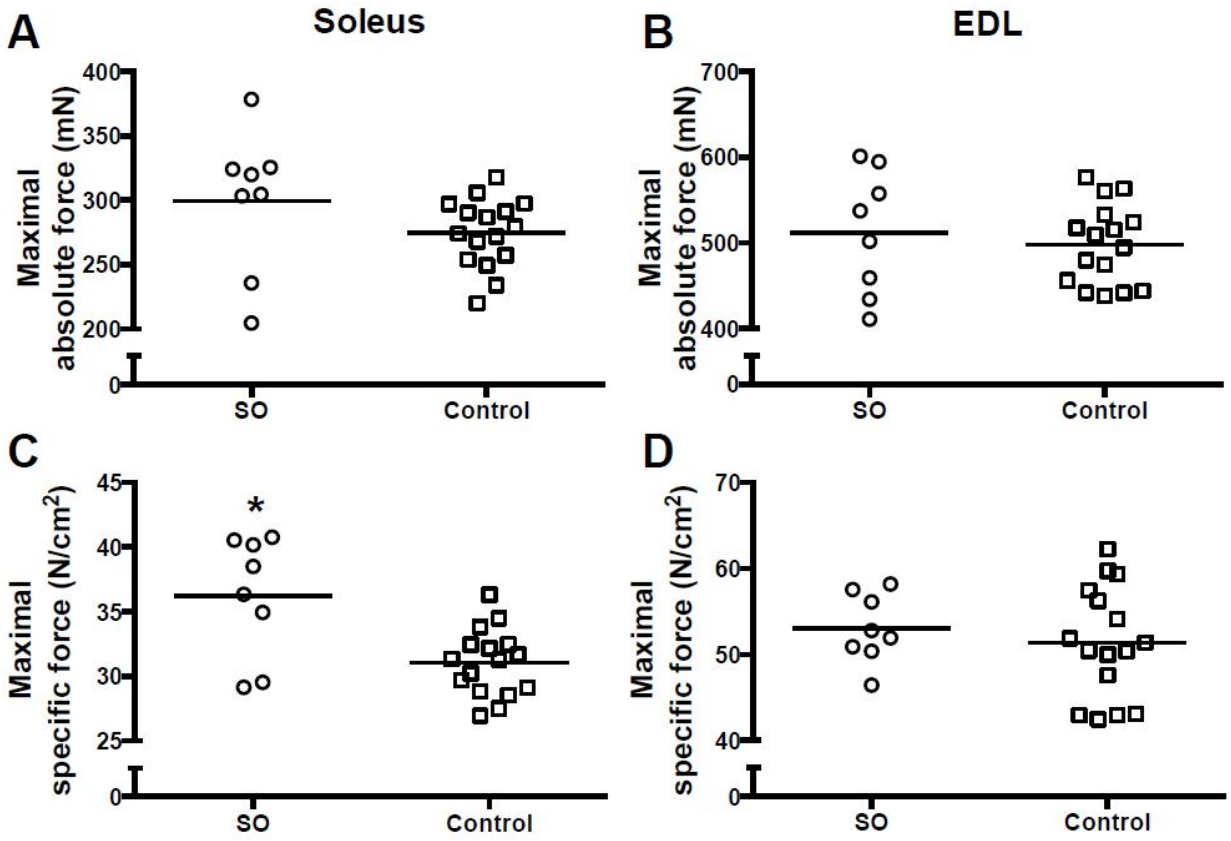

Figure 5.3: Individual and group mean maximal absolute (*panels A and B*) and specific (*panels C and D*) force of the soleus (*left panels*) and EDL (*right panels*) muscles in sham-operated animals (SO) and in the group subjected to femoral artery ligation and treated with the control regimen. Group comparisons were performed using Student's t-test. Values are means ± SE. * $p<0.05$ vs. Control. N=8 in the sham-operated group and 16 in the ligated, control group.

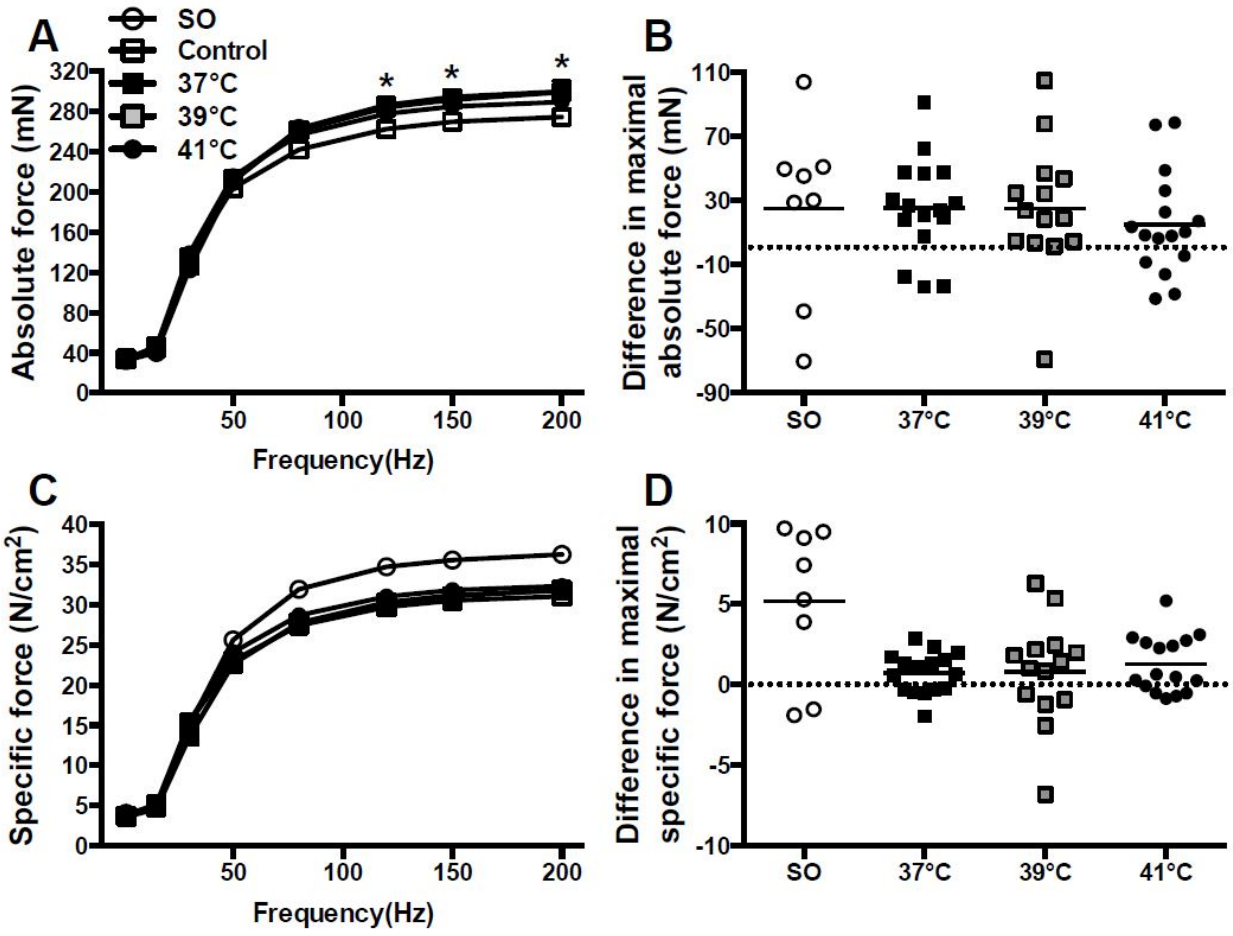

Figure 5.4: Absolute (*panel A*) and specific (*panel C*) force-frequency relationships of the soleus muscle. The groups subjected to femoral artery ligation were compared using a two-way repeated measures ANOVA. Dunnett's post-hoc test was performed when appropriate. *Panels B and D*: Individual and group mean differences in maximal absolute (*panel B*) and specific (*panel D*) force from the mean values obtained in the group subjected to femoral artery ligation and treated with the control regimen (dashed line). Values are means ± SE. * $p<0.05$ 37ºC and 39ºC vs. Control. N=8 in the sham-operated group (SO) and 14-16 in the Control, 37ºC, 39ºC, and 41ºC groups.

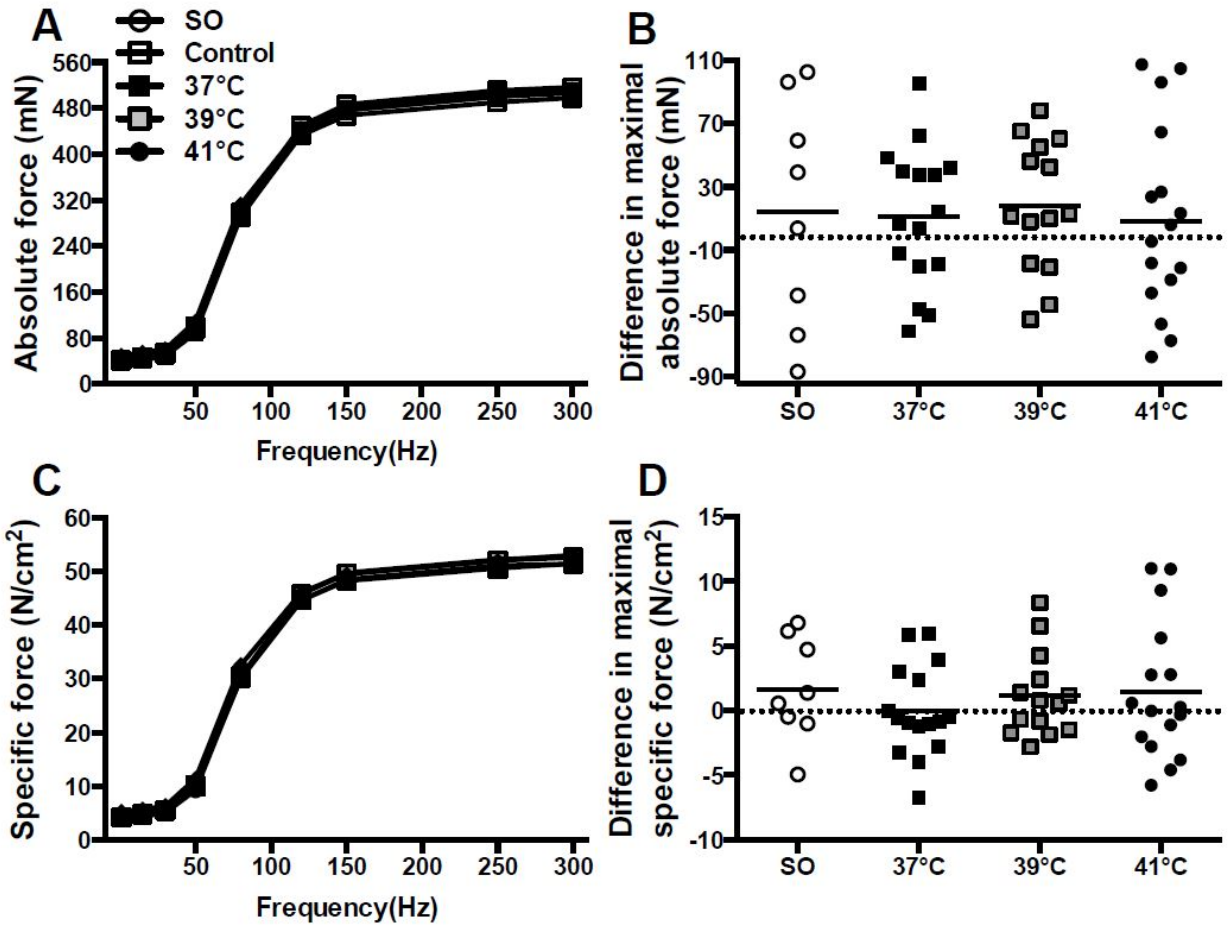

Figure 5.5: Absolute (*panel A*) and specific (*panel C*) force-frequency relationships of the EDL muscle. The groups subjected to femoral artery ligation were compared using a two-way repeated measures ANOVA. Dunnett's post-hoc test was performed when appropriate. *Panels B and D*: Individual and group mean differences in maximal absolute (*panel B*) and specific (*panel D*) force from the mean values obtained in the group subjected to femoral artery ligation and treated with the control regimen (dashed line). Values are means ± SE. N=8 in the sham-operated group (SO) and 14-16 in the Control, 37ºC, 39ºC, and 41ºC groups.

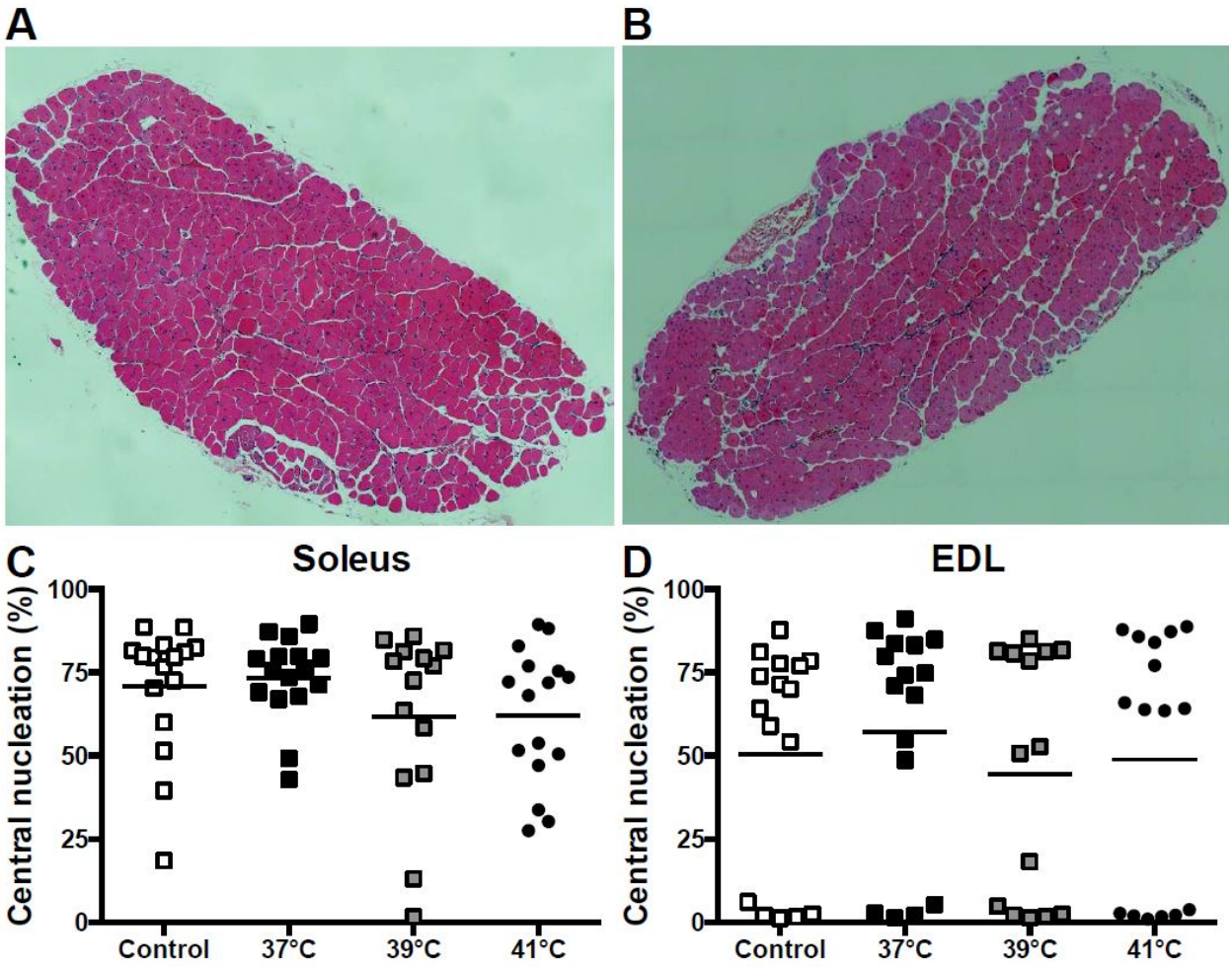

Figure 5.6: *Panel A*: Representative H&E-stained cross-sections of the soleus (*panel A*) and EDL (*panel B*) muscles. *Panels C and D*: Individual and group mean percentage of regenerating myofibers in each treatment group in the soleus (*panel C)* and EDL (*panel D*) muscles. Group comparisons were performed using a one-way ANOVA. Values are means ± SE. N=14-16 per group.

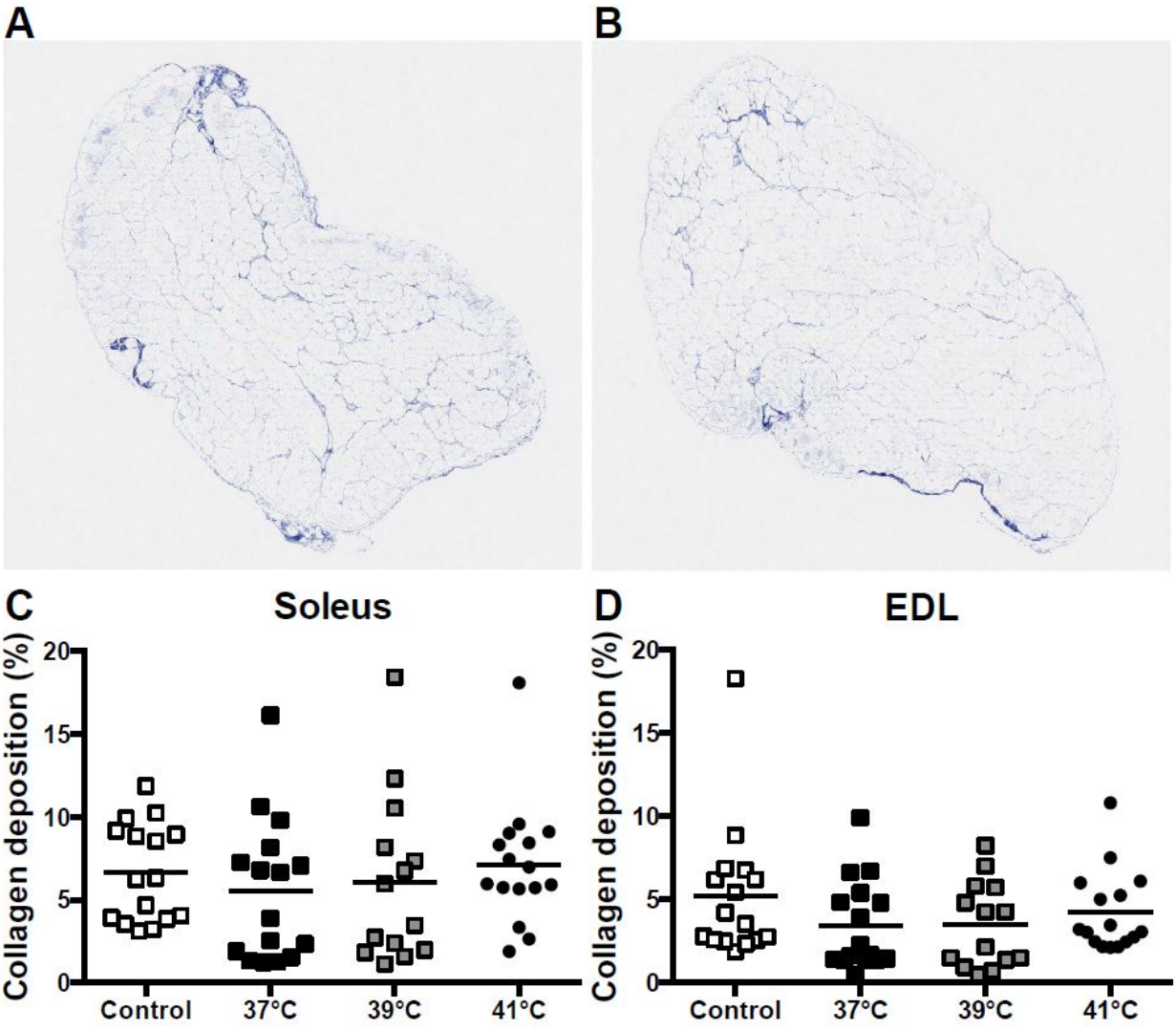

Figure 5.7: *Panel A*: Representative cross-sections of the soleus (*panel A*) and EDL (*panel B*) muscles stained with Masson's trichrome method to visualize collagenous connective tissue fibers. *Panels C and D*: Individual and group mean percentage of the total tissue area occupied by Masson's trichrome-stained tissue in the soleus (*panel C)* and EDL (*panel D*) muscles. Group comparisons were performed using a one-way ANOVA. Values are means ± SE. N=14-16 per group.

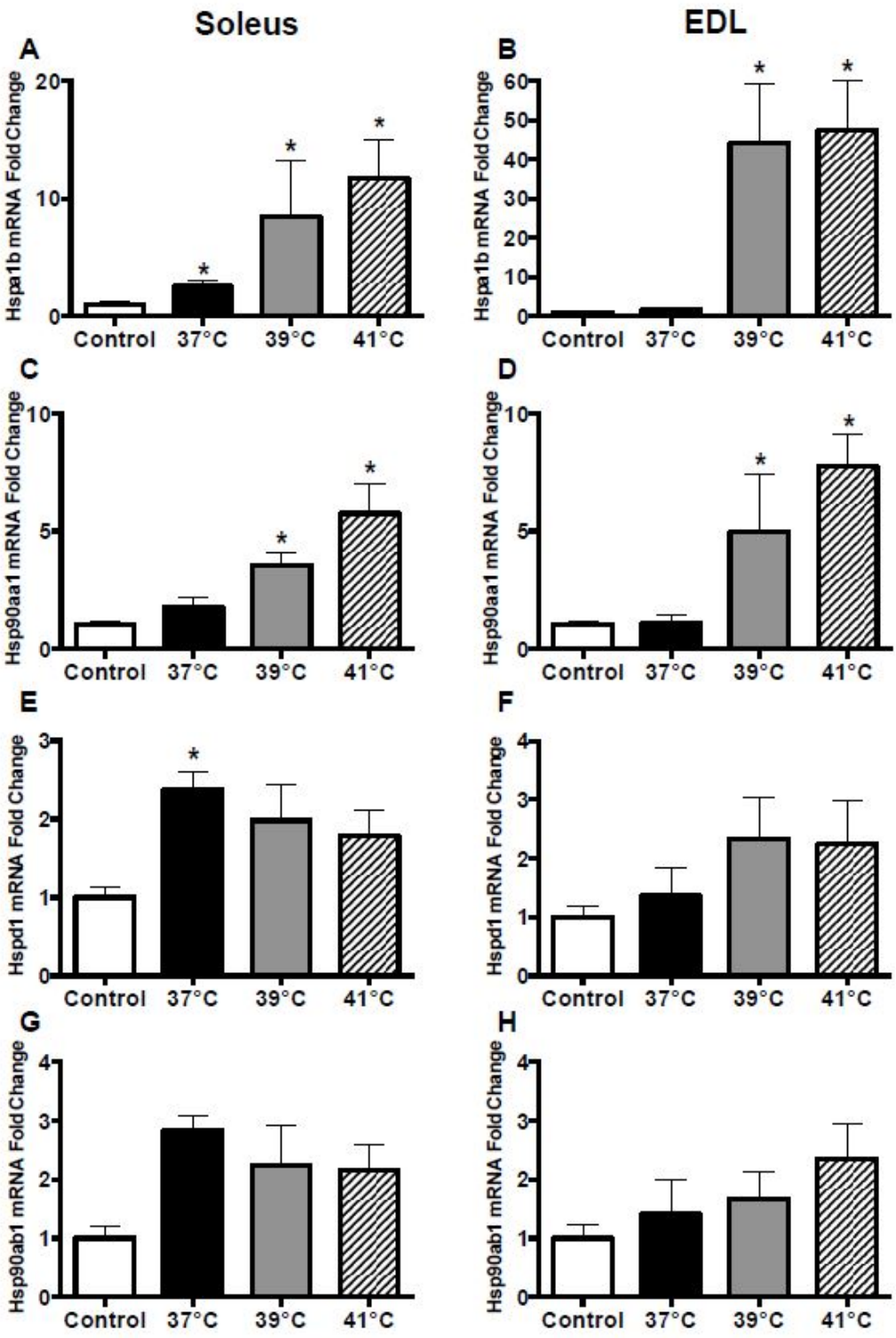

Figure 5.8: Changes in mRNA expression relative to the control intervention of Hspa1b (*panels A and B*), Hsp90aa1 (*panels C and D*), Hspd1 (*panels E and F*) and Hsp90ab1 (*panels G and H*) in the soleus (*left panels*) and EDL (*right panels*) muscles after exposure to a single session of HT. Group comparisons were performed using a one-way ANOVA. Dunnett's post-hoc test was performed when appropriate. Values are means ± SE. * $p<0.05$ vs. Control. N=6 per group.

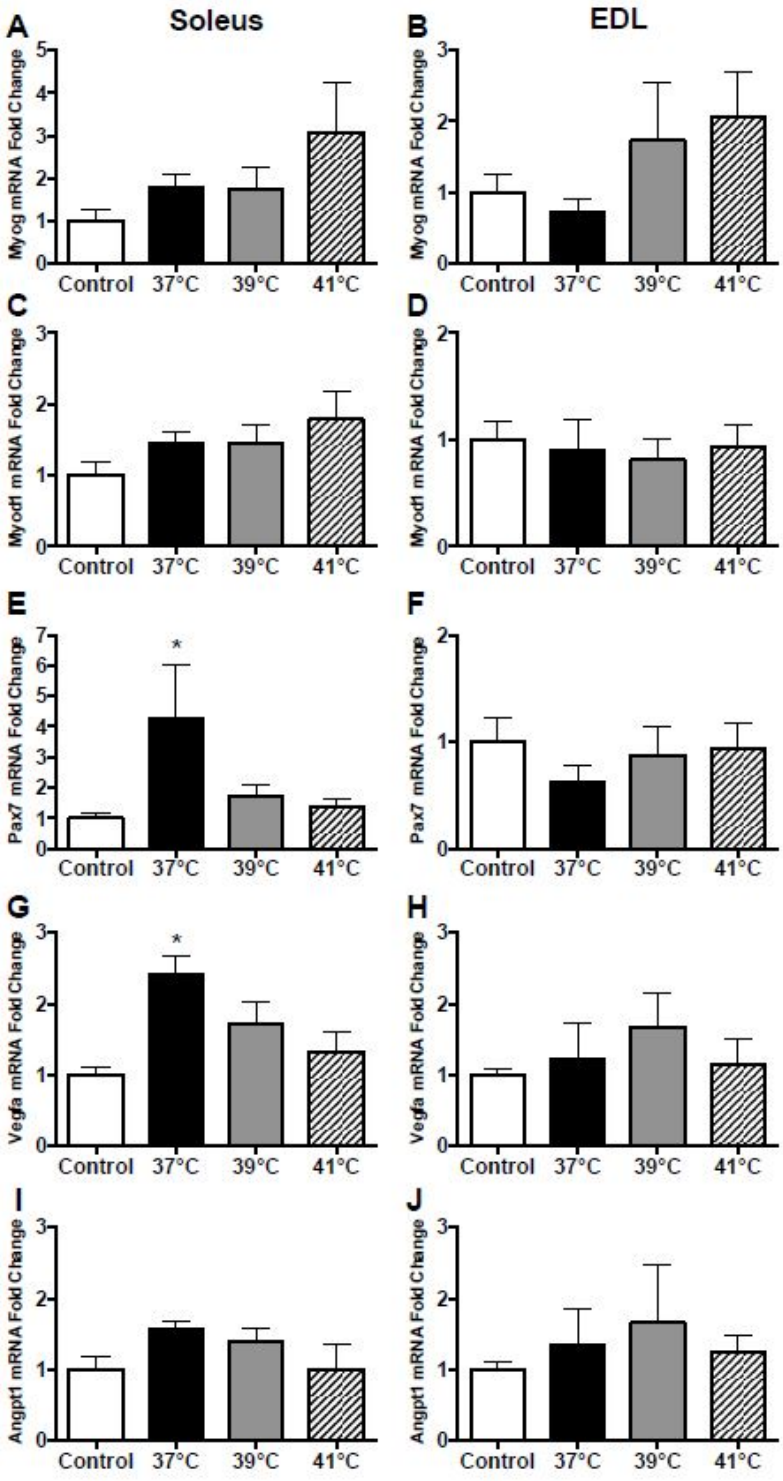

Figure 5.9: Changes in mRNA expression relative to the control intervention of Myog (*panels A and B*), Myod1 (*panels C and D*), Pax7 (*panels E and F*), Vegfa (*Panels G and H*) and Angpt1 (*Panels I and J*) in the soleus (*left panels*) and EDL (*right panels*) muscles after exposure to a single session of HT. Group comparisons were performed using a one-way ANOVA. Dunnett's post-hoc test was performed when appropriate. Values are means ± SE. * $p<0.05$ vs. Control. N=6 per group.

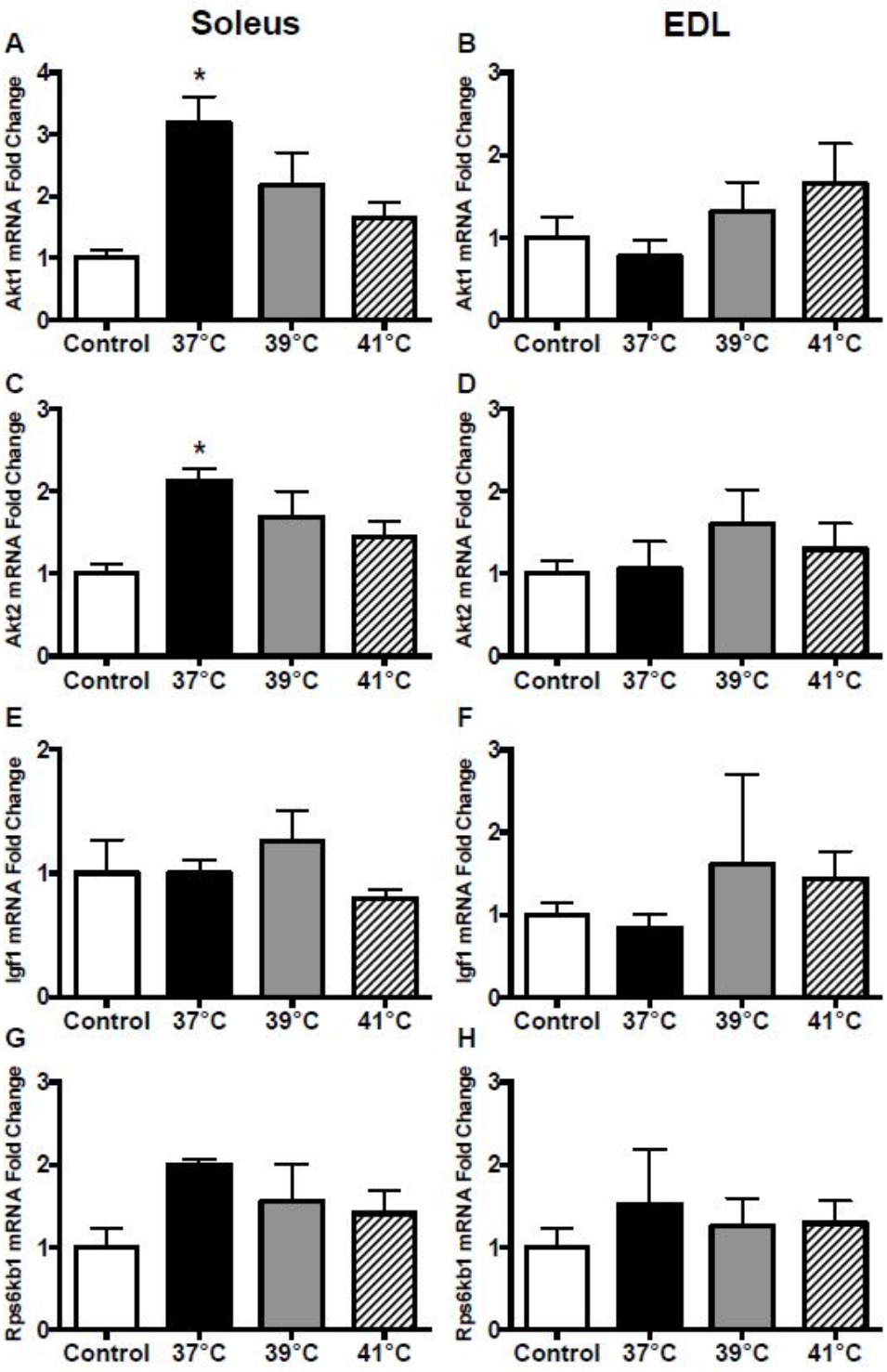

Figure 5.10: Changes in mRNA expression relative to the control intervention of Akt1 (*panels A and B*), Akt2 (*panels C and D*), Igf1 (*panels E and F*) and Rps6kb1 (*panels G and H*) in the soleus (*left panels*) and EDL (*right panels*) muscles after exposure to a single session of HT. Group comparisons were performed using a one-way ANOVA. Dunnett's post-hoc test was performed when appropriate. Values are means ± SE. * $p<0.05$ vs. Control. N=6 per group.

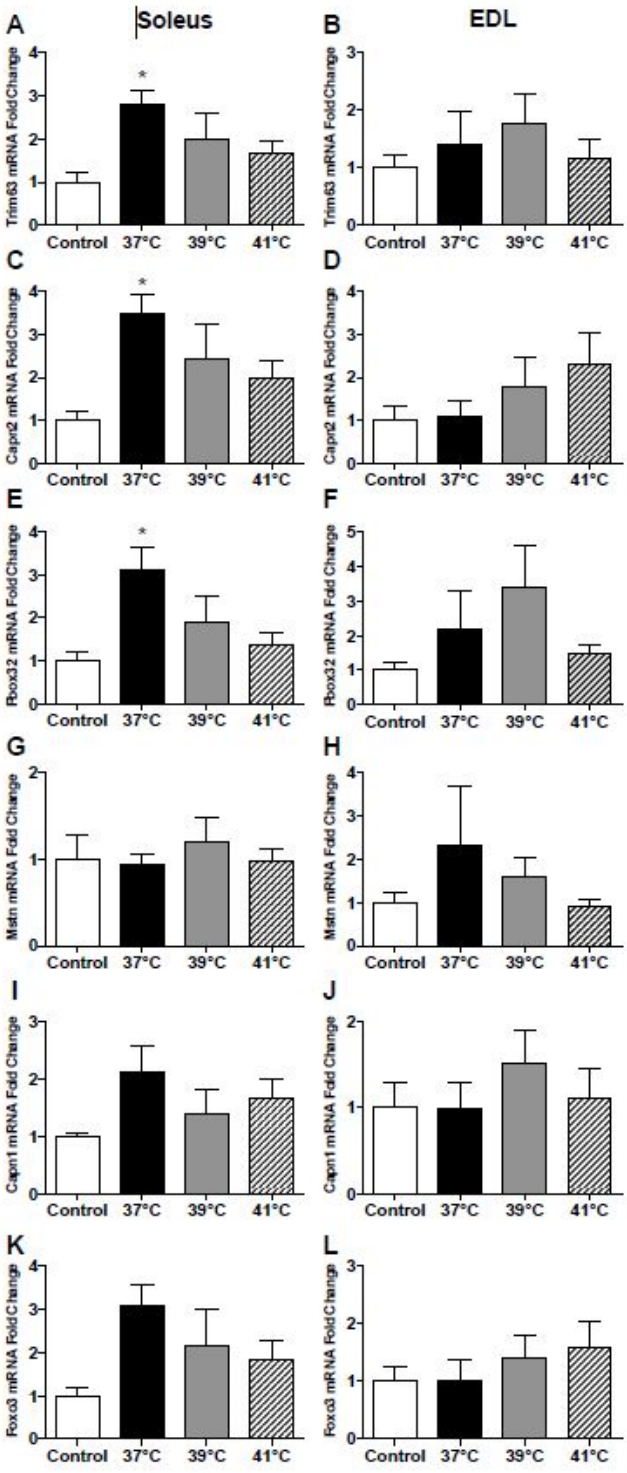

Figure 5.11: Changes in mRNA expression relative to the control intervention of Trim63 (*panels A and B*), Capn2 (*panels C and D*), Fbox32 (*panels E and F*), Mstn (*panels G and H*), Capn1 (*panels I and J*) and Foxo3 (*panels K and L*) in the soleus (*left panels*) and EDL (*right panels*) muscle after exposure to a single session of HT. Group comparisons were performed using a one-way ANOVA. Dunnett's post-hoc test was performed when appropriate. Values are means ± SE. * $p<0.05$ vs. Control. N=6 per group.

CHAPTER 6. CONCLUSION

The major goal of my dissertation studies was to determine the impact of heat therapy on skeletal muscle function and structure. Here we summarize the major findings of these studies, discuss the limitations and propose new directions for future studies.

Summary and overall conclusion

- Study 1

  Acute treatment with local heating for 90 min upregulated the mRNA expression of key angiogenic and myogenic mediators including vascular endothelial growth factor (VEGF) and angiopoietin 1 (ANGPT1) in human skeletal muscle. The increase in the the expression of these factors occurred without changes in core body temperature and mean arterial blood pressure compared to resting condition, thus highlighting the importance of local mechanism as primary mediators of heat-induced pro-angiogenic signaling.

- Study 2

  Maximal voluntary eccentric contractions of the knee extensors (300 repetitions) caused a significant reduction in muscle strength and fatigue resistance. The thigh exposed to repeated HT had greater fatigue resistance and increased protein expression of VEGF and ANGPT1 when compared to the thermonutral intervention. These findings provide strong evidence to support the clinical use of heat therapy to expedite recovery following muscle injury.

- Study 3

  Repeated local thigh heating improves peak isokinetic torque of the knee extensor muscle at weeks 4 (Control: 4.1±3.7 Nm vs. HT: 9±4.6 Nm) and 8 (Control: 1.7±2.7 Nm vs. HT: 7.7±2.9 Nm). Analysis of muscle biopsy specimens revealed that long-term exposure to heat therapy increased the content of eNOS and capillarization around type 2 muscle fibers compared to the control regimen. Overall, these observations indicate that heat therapy may be a potential therapeutic intervention to treat muscle weakness and capillary rarefaction observed in patients with chronic disease conditions.

- Study 4

  Bilateral ligation of femoral artery led to altered skeletal muscle morphology and impaired muscle function in a mouse model of ischemia-induced muscle damage. Two weeks after the surgery, animals were randomly allocated to receive HT (immersion in a water bath at 37°C, 39°C or 41°C) or control regimen for 30 min per session over 3 weeks. When compared with control group, maximal absolute force of the soleus muscle was significantly higher in animals that exposed to HT at 37°C and 39°C. Relative soleus muscle mass was also higher in the animals treated with HT at 39°C. These effects were not observed in the EDL muscle. Taken together, these findings suggest the beneficial effects of HT are muscle specific and dependent on the treatment temperature in a model of hindlimb ischemia.

Limitations and future directions

Primary functional outcomes from our human and animal studies suggest that repeated application of HT improves skeletal muscle strength and fatigue resistance. However, a major challenge we faced during these studies was that it is not possible to blind subjects to the exposure to heat. It is therefore conceivable that at least some of the beneficial effects may derive from a placebo effect. Another key limitation of our studies is that we have not measured muscle temperature during exposure to HT. In our human studies, we used a water-circulating garment to elicit local heat stress. This strategy is primarily designed to promote superficial, rather than deep tissue heating. It therefore imperative that future studies document the temporal changes in intramuscular temperature during treatment with this heat modality.

Molecular and morphological analysis of muscle biopsies reveal that acute and repeated application of heat therapy activate pro-angiogenic signaling and stimulate factors that mediate vascular growth. Both increased muscle temperature and blood flow likely explain our novel findings. However, the precise mechanisms by which heat therapy facilitates the angiogenic process was not determine in our studies. The use of cultured endothelial and/or muscle cells subjected to heat stress may be one viable model to explore in detail the fundamental mechanisms by which heat treatment promotes angiogenesis.