Heat acclimation increases mitochondrial respiration capacity of C2C12 myotubes and protects against LPS-mediated energy deficit

Meghan G. Patton¹ · Trevor L. Gillum² · Mandy C. Szymanski¹ · Lacey M. Gould¹ · Claire J. Lauterbach¹ · Roger A. Vaughan¹ · Matthew R. Kuennen¹

Received: 5 November 2017 / Revised: 14 March 2018 / Accepted: 15 March 2018 / Published online: 11 April 2018
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Abstract

This work investigated the effect of a 6-day heat acclimation (HA) protocol on myotube metabolic responses at baseline and in response to a subsequent lipopolysaccharide (LPS) challenge. C2C12 myotubes were incubated for 2 h/day at 40 °C for 6 days (HA) or maintained at 37 °C (C). Following 24-h recovery, myotubes were challenged with 500 ng/ml LPS for 2 h, then collected for analysis of protein markers of mitochondrial biogenesis and macronutrient storage. Functional significance of these changes was confirmed with mitochondrial respiration and glycolytic measurements on a Seahorse XF-96 analyzer. HA stimulated mitochondrial biogenesis and increased indicators of mitochondrial content [SIRT1 (+ 62%); PGC-1 α (+ 57%); NRF-1 (+ 40%); TFAM (+ 141%); CS (+ 25%); CytC (+ 38%); all $p < 0.05$]. Altered lipid biosynthesis enzymes [p-ACCA:ACC (+ 59%; $p = 0.04$) and FAS (− 86%; $p < 0.01$)] suggest fatty acid generation may have been downregulated, whereas increased GLUT4 (+ 69%; $p < 0.01$) and LDH-B (+ 366%; $p < 0.01$) suggest aerobic glycolytic capacity may have been improved. Mitochondrial biogenesis signaling in HA myotubes was suppressed by 500 ng/ml LPS (PGC-1 α , NRF-1, TFAM; all $p > 0.05$) but increased LDH-B (+ 30%; $p = 0.02$) and CPT-1 (+ 55%; $p < 0.01$) suggesting improved catabolic function. Basal respiration was increased in HA myotubes (+ 8%; $p < 0.01$) and HA myotubes maintained elevated basal respiration during LPS challenge (+ 8%; $p < 0.01$). LPS reduced peak respiration in C myotubes (− 6%; $p < 0.01$) but did not impair peak respiration in HA myotubes ($p > 0.05$). Oxidative reliance was elevated in HA over that in control (+ 25%; $p < 0.01$) and in HA + LPS over C + LPS (+ 30%; $p < 0.01$). In summary, HA stimulated mitochondrial biogenesis in C2C12 myotubes. HA myotubes exhibited (1) elevated basal/peak mitochondrial respiration capacities; (2) greater oxidative reliance; and (3) protection against LPS-mediated respiration impairment. Collectively, these data suggest HA may improve aerobic metabolism in skeletal muscle and protect against LPS-mediated energy deficit.

Keywords Hyperthermia · Lipopolysaccharide · Oxidative metabolism · Oxidative reliance

NEW AND NOTEWORTHY

Heat acclimation (HA) promotes mitochondrial biogenesis and increased glycolytic substrate processing capacity in C2C12 myotubes. Subsequent exposure to LPS causes a rapid downregulation of mitochondrial biogenesis signals. However, elevated oxidative metabolism capacity is maintained. This may be another mechanism by which activation of the heat shock response helps afford protection in skeletal muscle.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s12192-018-0894-1>) contains supplementary material, which is available to authorized users.

✉ Matthew R. Kuennen
mkuennen@highpoint.edu

¹ Department of Exercise Science, High Point University, One University Parkway, High Point, NC 27268, USA

² Department of Kinesiology, California Baptist University, Riverside 92504, CA, USA

Abbreviations

p-ACC	Phosphorylated acetyl-CoA carboxylase
ACC	Acetyl-CoA carboxylase
p-AMPK	Phosphorylated 5'-AMP-activated protein kinase
AMPK	5'-AMP-activated protein kinase
CPT	Carnitine palmitoyltransferase I
CS	Citrate synthase
CytC	Cytochrome c
FAS	Fatty acid synthase
FOXO1	Forkhead in rhabdomyosarcoma
GLUT4	Glucose uptake transporter 4
GS	Glycogen synthase
p-GSK-3 α	Phosphorylated glycogen synthase kinase 3 alpha

p-GSK-3 β	Phosphorylated glycogen synthase kinase 3 beta
GSK-3 α/β	Glycogen synthase kinase 3 alpha/beta
LDH-A	Lactate dehydrogenase A
LDH-B	Lactate dehydrogenase B
MYH4	Myosin heavy chain IIB
MyoD	Myocyte differentiation factor
TFAM	Mitochondrial transcription factor A
TNC	Troponin C
NRF-1	Nuclear respiratory factor 1
PGC-1 α	Peroxisome proliferator-associated receptor gamma co-activator-1
PPAR β/δ	Peroxisome proliferator-associated receptor beta/delta
PPAR γ	Peroxisome proliferator-associated receptor gamma
SIRT1	Sirtuin 1
UCP2	Uncoupling protein 2

Introduction

Repeated exposure to moderate levels of thermal stress (i.e., heat acclimation) promotes mitochondrial biogenesis in skeletal muscle via activation of AMP-activated protein kinase (AMPK) (Wu et al. 1999). This response is coordinated by peroxisome proliferator-activated receptor γ co-activator-1 α (PGC-1 α), which is a primary target of AMPK (Liu and Brooks 2012). Following phosphorylation by AMPK, PGC-1 α translocates into the nucleus and promotes expression of nuclear respiratory factor 1 (NRF-1) (Brenmoehl and Hoeflich 2013). The resultant increase in NRF-1 expression coordinates the induction of mitochondrial transcription factor A (TFAM) (Ryan and Hoogenraad 2007) and cytochrome C (Cyt C) oxidase (Herzig et al. 2000). TFAM regulates mitochondrial DNA replication and transcription (Clayton 1991), while Cyt C functions as the final cytochrome complex in the electron transport chain (Hawthorne and Harrison 1939). In addition to the above, AMPK also increases cellular NAD⁺ levels and sirtuin 1 (SIRT1) activity. SIRT1 has been shown to deacetylate PGC-1 α , increasing its activation (Canto et al. 2009).

The physiological significance of elevated temperature-mediated mitochondrial biogenesis is that it enhances cellular oxidative phosphorylation capacity. In turn, this helps to offset the elevated energetic requirements that are associated with hyperthermia exposure (Starkie et al. 1999). However, in vitro experiments utilizing C2C12 myotubes as a model system suggest repeated hyperthermia exposure may provide additional benefits in skeletal muscle. In these studies, repeated exposure to modest heat stress (39–41 °C) for short

durations (1–3 h) has been shown to promote increased myotube proliferation (Ohno et al. 2011) and mitochondrial content (Liu and Brooks 2012), in conjunction with shifts in myosin heavy chain (MHC) expression that are suggestive of a fast to slow twitch myotube phenotype conversion (Yamaguchi et al. 2010). In addition to PGC-1 α , we speculate that nuclear receptor peroxisome proliferator-activated receptor β/δ (PPAR β/δ), which is recognized as a key transcription factor in skeletal muscle biology, may play a role in these changes. Although not directly linked to hyperthermia exposure, we have previously shown that elevations in cellular PPAR β/δ content promote mitochondrial biogenesis (Schnuck et al. 2016). Others have shown that PPAR β/δ stimulates skeletal muscle fiber-type adaptations that are associated with improved endurance performance (Wang et al. 2004), with some of these effects being attributed to PPAR β/δ -mediated activation of PGC-1 α (Schuler et al. 2006).

If hyperthermic stress was shown to increase PPAR β/δ content in skeletal muscle, this might also confer important benefits on human health. Our group questions whether a hyperthermia-mediated increase in PPAR β/δ content might improve skeletal muscle capacity to maintain metabolic function during times of energetic stress. This would be useful in animal (Horowitz 2016) and human (Amorim et al. 2015; Kuennen et al. 2011) models of heat acclimation (HA), where 1 to 2 weeks of repeated thermal exposure is used to stimulate acquisition of the HA phenotype. It could also prove interesting in the context of lipopolysaccharide (LPS) exposure, which significantly increases cellular energy requirements (Frisard et al. 2010; Park and Jeoung 2016). If shown, this could be another potential mechanism by which HA affords protection against exertional heat stroke (EHS), the pathogenesis of which is driven by elevated concentrations of LPS in circulation (Kuennen et al. 2011). In line with this possibility, in our prior work, we have shown that a 7-day HA protocol activates the heat shock response and reduces circulating LPS concentrations in healthy, young males (Kuennen et al. 2013).

PPAR β/δ is the predominant PPAR isoform expressed in skeletal muscle (Yang et al. 2006). However, skeletal muscle cells also express PPAR γ , which plays a role in lipid metabolism. In contrast to the genes activated by PPAR β/δ , which stimulate fatty acid uptake and β -oxidation, the genes activated by PPAR γ stimulate lipogenesis (Dressel et al. 2003; Ehrenborg and Krook 2009). PPAR β/δ also enhances the expression of FOXO1, a transcription factor that targets genes promoting lipolysis, glycogenolysis, and gluconeogenesis. FOXO1 has also been shown to repress adipogenesis via binding to the promotor region for PPAR γ (Armoni et al. 2006). In addition to changes in transcript activity, cellular capacity for energy storage and mobilization is dictated by changes in metabolic enzyme content. For example, cellular capacity for glycogen

synthesis is dictated by glycogen synthase (Wu et al. 1999), the terminal enzyme in glycogen biosynthesis, as well as its major regulator, glycogen synthase kinase 3 (GSK-3). GSK-3 exists in two formats (GSK-3 α and GSK-3 β), both of which can be stimulated by tyrosine phosphorylation and inhibited by serine/threonine phosphorylation (Eldar-Finkelman 2002). Master regulators of lipid biogenesis include fatty acid synthase (FAS), acetyl-CoA carboxylase, and carnitine palmityl transferase (CPT) (Ameer et al. 2014). FAS is a multi-enzyme protein that catalyzes the terminal step in fatty acid biosynthesis. ACC is a multifunctional enzyme system that catalyzes the carboxylation of acetyl-CoA to malonyl-CoA, which transports α -ketoglutarate into the mitochondrial matrix to facilitate fatty acid chain synthesis. Malonyl-CoA can also inhibit the carnitine shuttle (rate limiter of β -oxidation) by interacting with CPT and preventing its association with fatty acids (McGarry et al. 1977).

The present study was conducted to answer three questions. First, given the prior report of increased mitochondrial biogenesis in C2C12 myotubes following repeated thermal stress exposure (Liu and Brooks 2012), we wanted to determine if the metabolic changes these authors reported at the mRNA level extended to quantifiable changes in protein content and functional changes in oxidative metabolism. Second, we wanted to determine if HA alters cellular content of PPARs and/or the major enzymatic regulators of lipid and glycogen biosynthesis, which could influence metabolic function through changes in the availability of oxidable substrate. Third, we wanted to determine if HA improves myotube capacity to maintain aerobic energy provision during subsequent challenge with a moderate dose (500 ng/ml) of LPS. Importantly, our work demonstrates that HA causes an increase in mitochondrial biogenesis in C2C12 myotubes, which translates to elevated mitochondrial respiration capacity and reduced susceptibility to LPS-mediated metabolic dysfunction.

Materials and methods

Cell culture

C2C12 mouse myoblasts from ATCC (Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose that was supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin. Cells were seeded in the growth media and maintained at 37 °C until they achieved 70–80% confluence. Differentiation of myoblasts into myotubes was accomplished by replacing growth media with DMEM supplemented with 2% horse serum and 100 U/mL penicillin/streptomycin for 6 days. Cells were grown in a humidified

37 °C incubator (NuAire NUANU4750E, Plymouth, MN, USA) with 5% supplemental CO₂ and media were routinely exchanged every 2 days. Unless otherwise stated, all experiments were performed using six replicates per treatment.

Heat acclimation protocol

Following differentiation, myotubes were maintained in the primary incubator under control conditions (37 °C/5% CO₂). One half of myotubes received mild heat stress exposure once daily for a period of 6 days. This was accomplished by transferring myotubes from the primary incubator to a secondary humidified incubator (ThermoFisher Scientific, Waltham, MA, USA) that received 5% supplemental CO₂ and was maintained at 40 °C. Using a YSI thermistor (accurate to 0.01 °C) that uploaded continuously to an external data logger (SQ2040; Grant Instruments Ltd, Cambridge, UK), it was determined that the secondary incubator required 7 min beyond cabinet door closing before returning to setpoint (40 °C). For that reason, each mild heat stress exposure was maintained for 127 min, after which myotubes were returned to the control incubator for a minimum of 24-h recovery prior to harvest or any subsequent experiment being performed. This repeated mild heat stress protocol (2 h/day of 40 °C exposure over 6 consecutive days) was utilized because it is known to mimic the intramuscular conditions that exist during a standard heat acclimation protocol (Watkins et al. 2008). In contrast to prior studies on HA in myotubes (Guo et al. 2016) (Liu and Brooks 2012), in the present protocol myotubes were differentiated for 6 days at 37 °C prior to the start of HA. This was done because following sexual maturity, mammalian skeletal muscle is known to be a highly stable tissue that exhibits little myonuclear turnover (Charge and Rudnicki 2004). This means that the majority of skeletal muscle cells that are exposed to thermal stress during a standard HA protocol are already fully differentiated. The benefit of this protocol is an increased generalizability of in vitro findings to the in vivo condition.

Heat acclimation protocol with subsequent LPS stimulation

The HA protocol was repeated on a separate set of myotubes. Following 24-h recovery, myotubes that had either received the 6-day HA or been maintained for the same duration under control conditions (37 °C) were challenged for 2 h with 500 ng/ml LPS (from *E. coli*) that was purchased from Santa Cruz Biotechnologies (sc-3535, Santa Cruz, CA, USA). The 500 ng/ml dose and 2-h duration of LPS challenge were selected because they have been shown to reduce the activity of citrate synthase and β -hydroxyacyl-CoA dehydrogenase in C2C12 myotubes, resulting in reduced fatty acid oxidation

and a reflexive increase in lactate production (Frisard et al. 2010). For reference, the authors of that manuscript reported similar metabolic findings in human primary skeletal muscle cells following 2-h LPS treatment at 500 ng/mL. Following LPS exposure, culture media were exchanged and cells were harvested (on ice) as described below.

Immunoblotting and protein expression

Whole cell lysates were prepared by harvesting cells on ice in RIPA buffer from Bio-Rad (Hercules, CA) supplemented with protease inhibitor mix (0.1%) from Bio-Rad (Hercules, CA), followed by incubation on ice for 1 h. Insoluble material was removed, and protein concentrations were determined by Bradford assay from Bio-Rad (Hercules, CA). Total protein (20–30 µg/sample) of treatment-pooled lysates was size-separated by 8–10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred to PVDF membranes. After blocking in TBST-5% non-fat milk powder for 1.25 h, membranes were probed at 4 °C overnight with target antibodies from Santa Cruz Biotechnologies (Santa Cruz, CA) as indicated in Table 1. Protein loading was confirmed with anti-β-actin primary antibody from Santa Cruz Biotechnologies (Santa Cruz, CA) at a dilution of 1:400 in TBST-5% non-fat milk powder. Bound antibodies were detected by horseradish peroxidase-conjugated secondary antibodies from Santa Cruz Biotechnologies (Santa Cruz, CA) at a dilution of 1:5000 in TBST-5% non-fat milk powder for 1 h at room temperature on an orbital platform at 250 rpm. Protein signal intensities were determined by chemiluminescence using the Clarity Western ECL substrate kit from Bio-Rad (Hercules, CA) and imaged using the ChemiDoc Touch from Bio-Rad (Hercules, CA). Relative signal intensities were normalized to β-actin and quantified using Image Lab from Bio-Rad (Hercules, CA).

Respiratory capacity and glycolytic function

An XF-96 analyzer from Agilent Seahorse Bioscience (Santa Clara, CA, USA) was used to perform mitochondrial bioenergetic assays that were based on published protocols (Wu et al. 2007). The XF assay medium (HCO₃[−] free modified DMEM, Seahorse Bioscience) was supplemented with 1 mM L-glutamine, 1 mM pyruvate, and further additions that were relevant to the experiment.

Cells were grown to confluence and differentiated for 6 days in Seahorse assay plates, after which time they either received the 6-day HA protocol or were maintained in differentiation media under control conditions. Twenty-four hours after HA myotubes had received their last 40 °C exposure, one half of the myotubes in each treatment were challenged with 500 ng/mL LPS for 2 h. Immediately following, the mitochondrial respiration test was performed by sequential addition of

2 µM oligomycin, 1.5 µM FCCP, and 0.5 µM rotenone/antimycin A to myotubes.

Using the XF cell Mitostress test kit (no. 103015-100; Seahorse Bioscience), we examined the following mitochondrial parameters: basal mitochondrial respiration (basal cellular respiration minus non-mitochondrial respiration), maximal respiratory capacity (maximal uncoupled respiration minus non-mitochondrial respiration), and non-mitochondrial respiration (rotenone/antimycin A-inhibited respiration). These results were expressed in pmol(O₂)min^{−1}. The extracellular acidification rate (ECAR) was measured in parallel with respiration analyses. From this, the following glycolytic parameters were established: basal ECAR and oligomycin-stimulated ECAR (peak glycolytic capacity). These results were expressed in mpHmin^{−1}.

Statistical analyses

All data are presented as group mean ± standard error of the mean. Protein content was analyzed by Student's *t* test. Respiratory capacity and glycolytic function data were analyzed by one-way ANOVA with Bonferroni's correction for pair-wise comparisons. GraphPad Prism 3.0 (La Jolla, CA, USA) was used for all statistical analyses, with significant differences determined by *p* < 0.05.

Results

Heat acclimation alone

Mitochondrial biogenesis and function As shown in Fig. 1, following the 6-day HA protocol, pAMPKα1 was increased by 80% (*p* < 0.01), SIRT1 was increased by 62% (*p* = 0.03), PGC-1α was increased by 57% (*p* < 0.01), NRF-1 was increased by 40% (*p* = 0.02), and TFAM was increased by 141% (*p* < 0.01). Markers of mitochondrial content were also elevated (CS + 25%; *p* = 0.04 and CytC + 38%; *p* = 0.04) and mitochondrial uncoupling protein 2 (UCP2) was reduced (−54%; *p* < 0.01), suggesting that HA may have contributed to improved mitochondrial capacity for energy provision. HA also contributed to a 71% increase in FOXO1 (*p* = 0.03), a 47% reduction in PPAR γ (*p* < 0.01), and a 35% elevation in PPAR β/δ (*p* = 0.02).

Lipid metabolism Elevated FOXO1 and PPAR β/δ in conjunction with reduced PPAR γ suggests 6-day HA may suppress lipid biogenesis capacity in C2C12 myotubes. For that reason, we next measured cellular levels of FAS, Ser221 phosphorylated ACC (p-ACC), and CPT. As shown in Fig. 2a, myotube FAS content was almost completely abolished (−86%; *p* < 0.01) following 6-day HA. This was shown in conjunction with a 59% increase in Ser221 phosphorylated ACC

Table 1 Summary of primary antibodies from Santa Cruz Biotechnologies (SCBT)

Primary antibody name	SCBT antibody identifier	SCBT number
Phosphorylated acetyl-CoA carboxylase	p-ACC β (Ser 219/Ser 221)-R	sc-30446-R
Acetyl-CoA carboxylase	ACC (H-76)	sc-30212
Phosphorylated 5'-AMP-activated protein kinase	p-AMPK α 1 (Ser 496)	sc-101631
5'-AMP-activated protein kinase	AMPK α 1/2 (D-6)	sc-74461
β -Actin	β -Actin (C4)	sc-47778
Carnitine palmitoyltransferase I	CPTI-M (M-14)	sc-20524
Citrate synthase	CS (G-3)	sc-390693
Cytochrome c	CytC (A-8)	sc-13156
Fatty acid synthase	FAS (H-300)	sc-20140
Forkhead in rhabdomyosarcoma	FKHR (H-128):	sc-11350
Forkhead in rhabdomyosarcoma-like 1	FKHRL1 (H-144)	sc-11351
Glucose uptake transporter 4	GLUT4 (H-61)	sc-7938
Glycogen synthase	GS1 (C-14)	sc-47188
p-Glycogen synthase kinase 3 α	p-GSK-3 α (Ser 21)	sc-101690
p-Glycogen synthase kinase 3 α/β	p-GSK-3 α/β (Tyr 279/Tyr 216)	sc-11758
Glycogen synthase kinase 3 α/β	GSK-3 α/β (0011-A)	sc-7291
Lactate dehydrogenase A	LDH-A (N-14)	sc-27230
Lactate dehydrogenase B	LDH-B (Q-21)	sc-133731
Mitochondrial transcription factor A	mtTFA (C-9)	sc-376672
Nuclear respiratory factor 1	NRF-1 (147.1)	sc-101102
Peroxisome proliferator-associated receptor gamma co-activator-1	PGC-1 (H-300)	sc-13067
Peroxisome proliferator-associated receptor alpha	PPAR α (H-2)	sc-398394
Peroxisome proliferator-associated receptor beta/delta	PPAR β/δ (F-10)	sc-74517
Peroxisome proliferator-associated receptor gamma	PPAR γ (B-5)	sc-271392
Sirtuin 1	SIRT1 (H-300)	sc-15404
Uncoupling protein 2	UCP2 (G-6)	sc-390189

($p = 0.04$), whereas CPT levels were unchanged ($p = 0.11$). These findings may be linked to elevated AMPK activity, which has been shown to increase Ser221 phosphorylation of ACC, resulting in inhibition of ACC and an overall lowering of fatty acid biosynthesis (Munday et al. 1988).

Glycolytic metabolism As shown in Fig. 2b, GLUT4 content was elevated by 69% ($p = 0.01$) following the 6-day HA protocol, which is suggestive of an increased capacity for glucose uptake. GS was not significantly altered ($p = 0.25$) following the 6-day HA protocol. However, we did note a significant increase in the total cellular content of GSK-3 α (+ 106%; $p = 0.02$) and GSK-3 α/β (+ 35%; $p < 0.01$). Thus, when serine phosphorylated GSK-3 α and tyrosine phosphorylated GSK-3 α/β were expressed relative to total cellular content of these proteins, we noted that the ratio of p-GSK-3 α to GSK-3 α was not altered by the 6-day HA protocol ($p = 0.08$), whereas the ratio of p-GSK-3 α/β to GSK-3 α/β was significantly reduced (− 50%; $p = 0.01$). Collectively, these data suggest that GSK-3-mediated inhibition of GS activity may have been

attenuated following 6-day HA, which could contribute to an elevation in glycogen biosynthesis.

To determine if glycolytic substrate metabolism was altered, we also measured LDH-A (which stimulates the conversion of pyruvate to lactate) and LDH-B (which stimulates the reverse conversion). As shown in Fig. 2c, LDH-A was not elevated by the 6-day HA protocol ($p = 0.06$), whereas LDH-B was significantly increased (+ 366%; $p < 0.01$). These data suggest that HA may confer improvements in aerobic glycolysis.

Heat acclimation with subsequent LPS stimulation

In summary, the cellular modifications that were made following the 6-day HA protocol included an increased mitochondrial biogenesis and content in conjunction with an increased content of glycogen biosynthetic enzymes and reduced regulators of lipid biosynthesis. Given that LPS has previously been shown to bias C2C12 myotubes towards anaerobic glycolysis (Frisard et al. 2010), we next wanted to determine if

Fig. 1 Heat acclimation stimulates mitochondrial biogenesis. **a** Phosphorylated 5'-AMP-activated protein kinase (p-AMPK) protein expression normalized to total AMPK and protein expression of NAD⁺-dependent deacetylase sirtuin 1 (SIRT1) in C2C12 myotubes following either 6-day hyperthermia protocol (40 °C) or cellular maintenance under control conditions (37 °C). **b** Peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α), nuclear respiratory factor 1 (NRF-1), and mitochondrial transcription factor A (TFAM) protein expression

following treatment of C2C12 myotubes as described above. **c** Citrate synthase (CS) cytochrome c (CytC), and uncoupling protein 2 (UCP2) protein expression following treatment of C2C12 myotubes as described above. **d** Forkhead box protein O1 (FOXO1), peroxisome proliferator-activated receptor γ (PPAR γ), and PPAR β/δ protein expression following treatment of C2C12 myotubes as described above. Unless otherwise indicated, target protein expression was normalized to β-actin as loading control. * indicates $p < 0.05$ compared with normothermic (37 °C) control

the 6-day HA protocol contributed to improved maintenance of oxidative metabolic function in response to moderate dose (500 ng/mL) LPS stimulation.

Mitochondrial biogenesis and function As compared to control myotubes that received LPS stimulation, myotubes that had received the 6-day HA prior to LPS stimulation exhibited

Fig. 2 Heat acclimation reduces enzymes associated with fatty acid biosynthesis but increases proteins associated with glucose uptake, glycogen biosynthesis, and oxidative glycolytic metabolism. **a** Fatty acid synthase (FAS), carnitine palmitoyltransferase 1 (CPT1), and phosphorylated acetyl-CoA carboxylase (p-ACC: normalized to total ACC) protein expression in C2C12 myotubes following either 6-day hyperthermia protocol (40 °C) or maintenance of cells under control conditions (37 °C). **b** Glucose uptake transporter 4 (GLUT4), glycogen synthase (GS), phosphorylated glycogen synthase kinase 3 alpha (p-

GSK-3α) protein expression expressed relative to total GSK-3α content, and phosphorylated glycogen synthase kinase 3 beta (p-GSK-3α/β) protein expression expressed relative to total GSK-3α/β content following treatment of C2C12 myotubes as described above. **c** Lactate dehydrogenase A (LDH-A) and lactate dehydrogenase B (LDH-B) protein expression following treatment of C2C12 myotubes as described above. Unless otherwise indicated, target protein expression was normalized to β-actin as loading control. * indicates $p < 0.05$ compared with normothermic (37 °C) control

57% higher levels of pAMPK α ($p=0.04$) and 31% higher levels of SIRT1 ($p<0.01$) (Fig. 3). Intriguingly, the elevations in PGC-1 α , NRF-1, and TFAM that had been observed following the 6-day HA protocol (described above) were shown to be completely abolished by the 2-h LPS exposure. While these data suggest that the HA-mediated stimulation of mitochondrial biogenesis was rapidly abrogated by subsequent LPS exposure, it is important to note that mitochondrial content remained elevated (as indicated by the maintenance of a 74% increase ($p=0.02$) in CytC content). Also similar to what had been shown following the 6-day HA alone, myotubes that received 6-day HA prior to LPS stimulation also exhibited a 32% reduction in UCP2 ($p=0.02$), a 74% elevation in FOXO1 ($p=0.02$), and a 42% elevation in PPAR β/δ ($p=0.04$). The observation that pAMPK α , SIRT1, UCP2, FOXO1, and PPAR β/δ expressions were similar following HA (regardless of LPS exposure) suggests that these adaptations may be foundational for HA. However, unlike what had been shown above, the PPAR γ content of HA myotubes that received LPS stimulation was not different ($p>0.05$) from control LPS-stimulated myotubes.

Lipid metabolism The lack of repressed PPAR γ signaling in HA myotubes suggests that 2-h LPS stimulation may have improved their lipid biogenesis capacity, as compared to HA myotubes in the absence of LPS stimulation. Intriguingly, following the 2-h LPS stimulation, the FAS

content of HA myotubes was also no longer reduced ($p>0.05$) (Fig. 4). Further, HA with subsequent LPS stimulation was also shown to reduce cellular content of Ser219 phosphorylated ACC (−33%; $p=0.02$) and total ACC (−35%; $p<0.01$). Thus, when phosphorylated ACC was expressed relative to total ACC, the difference (10%) was no longer significant ($p=0.28$). LPS administration has been shown to directly stimulate neutral lipid accumulation in cells (Victorov et al. 1989) via activation of toll-like receptor 4 (Frisard et al. 2010). As such, it is likely that the recovery of repressed PPAR γ signaling and the return of FAS to normal levels may help to confer this response in HA myotubes. However, we also noted a 55% increase in CPT ($p<0.01$), suggesting an increased mitochondrial capacity for FA uptake and the facilitation of β -oxidation. This increase in CPT may be necessary to ensure HA myotubes are able to meet the increased energy demand associated with LPS exposure.

Glycolytic metabolism As shown in Fig. 4, the GLUT4 content of HA cells was not shown to be elevated following the 2-h LPS stimulation ($p=0.46$). GS content was also unchanged ($p=0.25$). However, unlike what had been shown following HA alone, there was no longer any difference in the total cellular content of GSK-3 α ($p=0.21$) or GSK-3 α/β ($p=0.13$) between control and HA myotubes that had been challenged with LPS.

Fig. 3 LPS stimulation diminishes mitochondrial biogenesis signals in thermally preconditioned myotubes but mitochondrial content is maintained. **a** Phosphorylated 5'-AMP-activated protein kinase (p-AMPK) protein expression normalized to total AMPK and protein expression of NAD⁺-dependent deacetylase sirtuin 1 (SIRT1) protein expression following 500 ng/mL LPS stimulation for 2 h in C2C12 myotubes that had previously received either the 6-day hyperthermia protocol (40 °C) or been maintained for the same duration under control conditions (37 °C). **b** Peroxisome proliferator-activated receptor γ co-activator 1 α (PGC-1 α), nuclear respiratory factor 1 (NRF-1), and

mitochondrial transcription factor A (TFAM) protein expression following treatment of C2C12 myotubes as described above. **c** Citrate synthase (CS) cytochrome c (CytC), and uncoupling protein 2 (UCP2) protein expression following treatment of C2C12 myotubes as described above. **d** Forkhead box protein O1 (FOXO1), peroxisome proliferator-activated receptor γ (PPAR γ), and PPAR β/δ protein expression following treatment of C2C12 myotubes as described above. Unless otherwise indicated, target protein expression was normalized to β -actin as loading control. * indicates $p<0.05$ compared with normothermic (37 °C) control

Fig. 4 LPS stimulation may contribute to restoration of lipid storage capacity in HA myotubes but reduces protein markers that are associated with glucose uptake and glycogen biosynthesis capacities. **a** Fatty acid synthase (FAS), carnitine palmitoyltransferase 1 (CPT1), and phosphorylated acetyl-CoA carboxylase (p-ACC: normalized to total ACC) protein expression following 500 ng/mL LPS stimulation for 2 h in C2C12 myotubes that had previously received either the 6-day hyperthermia protocol (40 °C) or been maintained for the same duration under control conditions (37 °C). **b** Glucose uptake transporter 4 (GLUT4), glycogen synthase (GS), phosphorylated glycogen synthase kinase 3

alpha (p-GSK-3α) protein expression expressed relative to total GSK-3α content, phosphorylated glycogen synthase kinase 3 beta (p-GSK-3α/β) protein expression expressed relative to total GSK-3α/β content following treatment of C2C12 myotubes as described above. **c** Lactate dehydrogenase A (LDH-A) and lactate dehydrogenase B (LDH-B) protein expression following treatment of C2C12 myotubes as described above. Unless otherwise indicated, target protein expression was normalized to β-actin as loading control. * indicates $p < 0.05$ compared with normothermic (37 °C) control

While these data indicate that both HA and LPS are capable of stimulating an increase in the GSK-3 content of fully differentiated C2C12 myotubes, it is noteworthy that the phosphorylation status of GSK-3 proteins in HA myotubes was shown to be the inverse of what had been observed following HA alone. Specifically, serine phosphorylation of GSK-3α (which indicates repression of GSK-3 activity) was now significantly depressed (-87% ; $p = 0.02$), whereas tyrosine phosphorylation of GSK-3α/β (which indicates GSK stimulation) was now shown to be elevated ($+460\%$; $p = 0.03$). This would suggest an overall lowering of GS capacity for glycogen synthesis following LPS stimulation in HA myotubes, as compared to control myotubes that had also received LPS stimulation. The aerobic glycolytic metabolism capacity of HA myotubes did not appear to be depressed by the LPS challenge, as no change was shown in LDH-A ($p = 0.22$) and LDH-B was shown to be increased by 30% ($p = 0.02$).

Functional significance of HA and metabolic responses to subsequent LPS exposure

To assess the functional significance of changes in metabolic protein content following HA alone as well as HA with subsequent LPS exposure, we next examined

myotube mitochondrial respiration and glycolytic function. As shown in Fig. 5, HA myotubes exhibited an 8.4% increase in basal respiration over control cells. Following LPS stimulation, HA cells retained a 7.9% elevation in basal respiration over control (both $p < 0.05$). Peak respiratory capacity was also elevated by 2.9% in HA cells over control. Interestingly, while LPS administration reduced peak respiration in control cells by 5.9% ($p < 0.05$), it did not impair peak respiration in HA cells ($p > 0.05$). Basal glycolytic metabolism in HA was 13.3% lower than that in control and basal glycolytic metabolism in HA myotubes that were challenged with LPS was 15.5% lower than that of control myotubes that received LPS (both $p < 0.05$). In contrast to basal glycolytic metabolism, there was no difference in peak glycolytic metabolism between conditions ($p = 0.127$). Therefore, although HA myotubes were biased towards mitochondrial respiration at baseline, their ability to achieve a high rate of glycolytic flux under stress conditions was not altered. The ratio of basal oxygen consumption to basal glycolytic metabolism (OCR:ECAR) provides an index of cellular oxidative reliance. As shown in Fig. 5d, oxidative reliance in HA was elevated by 25.2% over control and oxidative reliance in HA + LPS was elevated by 29.5% over control + LPS (both $p < 0.05$). From these data, we conclude that

Fig. 5 Heat acclimation increases mitochondrial respiration and oxidative reliance in C2C12 myotubes. These changes are maintained following LPS stimulation. **a** Basal oxygen consumption following exposure to 6-day hyperthermia protocol [HA] or maintenance under control conditions [C], where following a 24-h recovery period one half of myotubes in each condition received 500 ng/mL LPS stimulation for 2 h [HA + LPS and C + LPS; respectively]. * indicates $p < 0.05$ as compared to HA. **b** Peak oxygen consumption following treatment of C2C12 myotubes as described above. * indicates difference between heat conditions; $p < 0.05$. ** indicates difference within conditions; $p < 0.05$. **c** Basal glycolytic metabolism (indicated by extracellular acidification rate

or ECAR) following treatment of C2C12 myotubes as described above. * indicates difference between heat conditions; $p < 0.05$. **d** Oxidative reliance (expressed as OCR:ECAR) from basal metabolic conditions following treatment as described above. * indicates difference between heat conditions; $p < 0.05$. The experiments depicted in panels **a–d** were conducted on two plates that were run consecutively in a XF-96 analyzer. One plate received the 6-day hyperthermia protocol (40 °C). The other plate was maintained for 6 days under control conditions (37 °C). Following 24-h recovery, one half of the myotubes on each plate received 500 ng/mL LPS stimulation for 2 h. Basal and peak respiration and glycolytic measurements were taken immediately afterwards

2 h of LPS stimulation at 500 ng/ml causes a reduction in cellular oxygen consumption and that 6 days of HA is sufficient stimulus to protect against this response.

Discussion

HA was shown to promote mitochondrial biogenesis in C2C12 myotubes via elevations in AMPK, SIRT-1, and PGC-1 α . As such, the results of the present study confirm the prior findings by Liu et al. (Liu and Brooks 2012). UCP2 was also shown to be downregulated following HA in the present study, suggesting that oxidative phosphorylation efficiency may have been improved. This could indicate a compensatory mechanism that helps myotubes offset the elevated biosynthetic requirements that are necessary to maintain mitochondrial biogenesis during HA. In addition to these changes in protein content, functional outcomes were also improved by HA. Specifically, basal and peak respiration

capacities were increased, in conjunction with a reduction in basal (but not peak) glycolytic metabolism. There was also evidence of reduced LPS-mediated suppression of peak respiratory capacity and an overall lower reliance on anaerobic glycolysis in HA myotubes.

These findings are important because skeletal muscle is one of the largest and most metabolically active organs in the human body during exertional heat stress (Pedersen and Febbraio 2012). Prior work has suggested that heat acclimation (HA) may increase the metabolic efficiency of skeletal muscle during submaximal exercise (Young et al. 1985). The elevations in mitochondrial biogenesis that were shown in the present study, which may enhance muscle cell ATP restitution capacity some 15-fold greater than anaerobic glycolysis alone, support this claim. Although we are the first to investigate the effect of HA on metabolic responses in differentiated C2C12 myotubes, other researchers have examined the effect of exposing C2C12 myotubes to HA during the differentiation period. One group differentiated myotubes in an incubator set to

Fig. 6 Summary of findings: C2C12 myotube responses to the 6-day HA protocol. Repeated exposure to low-grade thermal stress stimulates mitochondrial biogenesis in C2C12 myotubes via elevations in AMPK, SIRT1, and PGC-1 α . Myotube capacity for aerobic metabolism is also improved and myotubes become biased towards glycolytic function, possibly at the expense of lipid handling. HA-mediated improvements in mitochondrial respiration are maintained during subsequent LPS stimulation, but mitochondrial biogenesis signals are rapidly degraded. This

could represent a negative feedback mechanism whereby inactivation of mitochondrial biogenesis may help to prevent a bio-synthetically mediated energy deficit from compromising macronutrient availability. Dark ovals with green outline identify proteins that were significantly increased ($p < 0.05$) by the 6-day HA protocol. White ovals with red outline identify proteins that were significantly reduced ($p < 0.05$) by the 6-day HA protocol. Intermediate ovals with gray outline identify proteins that were not significantly altered ($p > 0.05$) by the 6-day HA protocol

37 °C for 5 days, transferring myotubes to a secondary incubator that was controlled at 40 °C for 1 h/day thermal stress exposures (Liu and Brooks 2012). Another group differentiated myotubes in an incubator set to 39 °C for 5 days (Guo et al. 2016). Data generated by these researchers provided evidence of a fast to slow myotube phenotype transition, in conjunction with an upregulation of markers of mitochondrial biogenesis that suggested a greater aerobic capacity.

In the present study, myotubes were differentiated for 6 days at 37 °C. Following this, differentiated myotubes were transferred to a second incubator that was controlled at 40 °C for 2 h/day thermal stress exposures. Given that HA has been shown to shift myotubes towards a more aerobic phenotype, it was possible that our findings may have only been generalizable to skeletal muscle that exhibits a prominent amount of slow twitch fibers. This was a concern because intact skeletal muscle exhibits an approximately equal distribution of slow and fast twitch fibers. To address this concern, we performed an additional experiment to determine if markers of myotube differentiation and skeletal muscle cell phenotype were altered in the differentiated myotubes that received our HA protocol. We did not note any differences in differentiation or muscle cell phenotype, suggesting the present study findings are not specific to one skeletal muscle fiber phenotype over another. Further information on this experiment is provided as a data supplement (Supplemental Table 1, Supplemental Figure 1).

Using a continuous hyperthermia protocol, Yamaguchi et al. (2010) were the first to report an increased myotube diameter and fusion index in C2C12 myotubes that had been differentiated for 3 days at 39 °C (Yamaguchi et al. 2010). Myotubes that received the continuous hyperthermia protocol also exhibited elevated myosin heavy chain (MHC) type I and decreased MHC type II, suggesting a fast to slow fiber-type shift in myoblasts that the authors subsequently attributed to activation of PGC-1 α (Yamaguchi et al. 2010). Subsequent to that study, another group replicated the continuous hyperthermia protocol in quail myoblasts and reported similar changes in myotube proliferation and MHC expression (Choi et al. 2016). A third report in this area provided evidence of elevated gene expression of PPAR γ in C2C12 myotubes that had been incubated for 5 days at 41 °C (Guo et al. 2016). Based on these manuscripts, we had anticipated that HA would increase myotube capacity for lipid processing and storage. However, FOXO1 and PPAR β/δ were shown to be elevated by HA in the present study, in conjunction with reductions in PPAR γ and FAS, suggesting that the opposite response may have occurred. We speculate that although intramuscular triglycerides may be preferential (over glycogen) in highly aerobic slow twitch muscle fibers, lipogenesis in the present model may have been depressed because available fatty acids were instead being shuttled into a catabolic pathway to meet the elevated energy demand that accompany stimulation of mitochondrial biogenesis.

Table 2 Pathways by which heat acclimation may confer metabolic cross-tolerance

Pathway	Protein	37 °C	40 °C	37 °C + LPS	40 °C + LPS
Mitochondrial biogenesis and energetic efficiency	p-AMPK/AMPK	99 ± 2%	194 ± 12% [†]	105 ± 16%	162 ± 17% [†]
	SIRT1	106 ± 9%	168 ± 21% [†]	103 ± 2%	134 ± 4% [†]
	PGC-1	103 ± 4%	160 ± 9% [†]	100 ± 1%	98 ± 10%
	NRF-1	93 ± 6%	133 ± 12% [†]	100 ± 2%	97 ± 5%
	mtTFA	111 ± 15%	252 ± 28% [†]	91 ± 10%	120 ± 14%
	CS	100 ± 5%	126 ± 10% [†]	97 ± 7%	114 ± 18%
	CytC	101 ± 5%	138 ± 15% [†]	105 ± 10%	179 ± 23% [†]
	UCP2	101 ± 4%	46 ± 3% [↓]	97 ± 4%	65 ± 14% [↓]
Energy mobilization and storage	FKHR	106 ± 11%	177 ± 24% [†]	109 ± 16%	182 ± 16% [†]
	PPAR β/δ	99 ± 7%	134 ± 9% [†]	96 ± 5%	138 ± 8% [†]
	PPAR γ	108 ± 4%	61 ± 2% [↓]	102 ± 9%	94 ± 7%
Lipid metabolism	FAS	94 ± 11%	8 ± 3% [↓]	83 ± 22%	66 ± 7%
	p-ACC/ACC	94 ± 11%	153 ± 22% [†]	98 ± 4%	108 ± 16%
	CPTI-M	100 ± 10%	82 ± 7%	99 ± 1%	154 ± 16% [†]
Glycolytic metabolism	GLUT4	98 ± 7%	168 ± 19% [†]	119 ± 22%	122 ± 28%
	p-GSK-3α/ GSK-3α	101 ± 9%	67 ± 10%	125 ± 38%	31 ± 7% [↓]
	p-GSK-3α/β / GSK-3α/β	105 ± 10%	55 ± 10% [↓]	130 ± 37%	588 ± 177% [†]
	LDH-B	124 ± 50%	490 ± 67% [†]	98 ± 3%	127 ± 9% [†]

Table reports changes in protein expression as compared to control conditions (37 and 37 °C + LPS; respectively). The direction of changes that were significant at $p \leq 0.05$ is demarcated by [†] or [↓] arrows. Major study findings are summarized below

Project Summary. HA causes increased energetic stress, which activating PGC1α and increases mitochondrial biogenesis. UCP2 is also downregulated, presumably in an effort to increase energetic efficiency. PPARβ/δ and FOXO1 are increased, whereas PPAR γ is reduced, suggesting a greater bias towards energy mobilization. Changes in these transcripts were shown in concert with elevated phosphorylation of ACC and reduced FAS, suggesting reduced cellular emphasis on lipid storage. In contrast, increased GLUT4 content and a reduced p-GSK-3α/β:GSK3α/β ratio suggest improved glucose uptake capacity and reduced GSK-3-mediated repression of glycogen synthase activity. LDH-B was also increased, suggesting a greater aerobic glycolytic capacity.

When HA myotubes were challenged with 2-h LPS exposure, we noted a rapid turn-off of mitochondrial biogenesis signaling, as indicated by lack of significant findings for PGC-1 α, NRF-1, and mtTFA in HA myotubes immediately following LPS exposure. In contrast, elevated levels of SIRT1 and p-AMPK were shown to be retained. LPS is known to increase cellular energy requirements, so we speculate energy mobilization signals were retained whereas mitochondrial biogenesis (which itself could contribute to a biosynthesis-mediated energy deficit) was abrogated. FOXO1 and PPAR β/δ also remained elevated, further supporting the need for energy mobilization. However, it appears that cellular macronutrient preference may have been altered by LPS exposure. Specifically, PPAR γ and FAS were no longer reduced and p-ACC was no longer elevated, suggesting less cellular repression lipid use as a substrate. An increase in CPT1 would be expected to improve fatty acyl CoA entry into the mitochondrion, presumably to facilitate β-oxidation. While LDH-B also remained elevated (suggesting aerobic glycolytic capacity was maintained), we also noted a significant reduction in the p-GSK-3α:GSK-3α ratio and a significant elevation in the ratio of p-GSK-3α/β:GSK-3α/β. Both of these changes in GSK-3 activity suggest glycogen synthase activity may have been suppressed.

These changes were also in line with functional assay observations, where HA improved C2C12 aerobic metabolism capacity and reduced reliance on anaerobic glycolysis. In response to LPS exposure, greater aerobic metabolic rate was retained, in concert with reduced reliance on basal glycolytic function and overall better maintenance of cellular metabolism capacity.

Given the evidence supporting a hyperthermia-mediated acceleration of myogenesis (Choi et al. 2016; Guo et al. 2016; Yamaguchi et al. 2010), we feel it is important to point out that myogenesis also contributes to a metabolic shift (from anaerobic glycolysis to oxidative phosphorylation) and an induction of mitochondrial biogenesis (Kraft et al. 2006). During myogenesis, mitochondrial biogenesis is coordinated through MyoD and activation of the non-canonical NF-κB pathway. Non-canonical NF-κB signaling has been implicated in the regulation of PGC-1β transcription, with NF-κB p52 being shown

to bind within the first intron of the PGC-1β gene, activating its transcription and resulting in stimulation of mitochondrial biogenesis and oxidative phosphorylation in skeletal muscle (Bakkar et al. 2012). Using a bioinformatics approach, Shintaku et al. (2016) reported that like PGC-1β, PPAR β/δ also contains MyoD binding peaks that are interspersed by p52 binding sites (Shintaku et al. 2016). Given that PPAR β/δ signals for fatty acid uptake and β-oxidation, it is plausible that alterations in non-canonical NF-κB signaling following HA may help to coordinate the mitochondrial biogenesis and

substrate mobilization that are necessary to maintain cellular function.

We are the first to report that HA protects against the LPS-mediated reduction in mitochondrial respiration capacity of C2C12 myotubes. However, parallels can be drawn between our “metabolic cross-tolerance” work in skeletal muscle cells and the “heat acclimation-mediated cross-tolerance” that has been reported by another group in cardiomyocytes (Assayag et al. 2012). Those authors characterized changes in molecular markers of mitochondrial biogenesis following short-term (2 days) and long-term (30 days) HA in rats. They also determined if HA-mediated changes in mitochondrial function afford protection against subsequent ischemia/reperfusion injury (Assayag et al. 2012). Both 2- and 30-day HA were shown to elevate PGC-1 α gene activity in cardiomyocytes. PPAR β/δ and NRF-1 were also shown to be upregulated following 2-day HA, whereas PPAR γ was shown to be downregulated following 30-day HA. These alterations in PGC-1 α , PPAR β/δ , NRF-1, and PPAR γ in cardiomyocytes match our findings in skeletal muscle cells. Interestingly, when cardiomyocytes in the Assayag et al. 2012 study were subjected to ischemia-reperfusion injury, PGC-1 α and NRF were shown reduced in control as well as the 2- and 30-day HA groups (Assayag et al. 2012). These data mirror what was shown in control and HA myotubes following LPS challenge in the present study, suggesting that independent of the stress imposed, cellular insult rapidly dampens signals coordinating mitochondrial biogenesis (Assayag et al. 2012). Mitochondria that were isolated from HA animals in the Assayag et al. 2012 study also exhibited higher levels of oxygen consumption and were better able to maintain mitochondrial membrane potential following the ischemia/reperfusion challenge. This offered protection against the loss of respiratory complex activity and an associated reduction in ATP production, which were shown in mitochondria that were isolated from control animals (Assayag et al. 2012). Those data are congruent with our findings, supporting the acquisition of “heat acclimation-mediated cross-tolerance” in our C2C12 model.

In addition, another group recently reported improved cell viability in C2C12 myotubes that received HA [3-h daily thermal exposure (39.5 °C) for 3 days] prior to a more severe thermal challenge (4 h at 43 °C) (Yu et al. 2016). The authors attributed this benefit to mitochondria biogenesis-mediated alterations in mitochondrial morphology (overly elongated mitochondria), which protected HA myotubes against apoptotic death that featured cytochrome C release from mitochondria and subsequent activation of caspase 3/7 cascades (Yu et al. 2016). As HA was shown to stimulate mitochondrial biogenesis in the present study, it is possible that similar changes in mitochondrial morphology could partially account for the protection that HA afforded myotubes against subsequent LPS exposure. Future research should be conducted to determine if HA myotubes exhibit reduced apoptotic and inflammatory cascades

following LPS stimulation. Our work, which demonstrates reduced susceptibility to LPS-mediated metabolic dysfunction in HA myotubes, will help to inform future research. We have generated a summary figure (Fig. 6) and a summary table (Table 2) that highlight our major study findings.

Acknowledgements We would like to thank the Department of Physical Therapy (Congdon School of Health Sciences) and the School of Arts and Sciences for the use of shared lab space and equipment.

Author contribution MGP made a significant contribution to the first draft of the manuscript and was lead author. MGP, MCS, LMG, and CJL assisted with experiments and manuscript preparation. TLG assisted with manuscript preparation. RAV designed and conducted experiments and assisted with manuscript preparation. MRK is corresponding author. He conceived the study, designed, conducted and oversaw all experiments, performed statistical analyses, oversaw MGP in drafting of the manuscript, and wrote subsequent versions of the manuscript as dictated by the reviewers. All authors read and approved the final manuscript.

Funding information Financial support for this work was provided by the Department of Exercise Science within the Congdon School of Health Sciences.

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