Heat acclimation increases inflammatory and apoptotic responses to subsequent LPS challenge in C2C12 myotubes

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Abstract

This work investigated the ability of a 6-day heat acclimation protocol to impart heat acclimation-mediated cross-tolerance (HACT) in C2C12 myotubes, as indicated by changes in inflammatory and apoptotic responses to subsequent lipopolysaccharide (LPS) challenge. Myotubes were incubated at 40 °C for 2 h/day over 6 days (HA) or maintained for 6 days at 37 °C (C). Following 24 h recovery, myotubes from each group received either no stimulation or 500 ng/ml LPS for 2 h (HA + LPS and C + LPS, respectively). Cell lysates were collected and analyzed for protein markers of the heat shock response, inflammation, and apoptosis. As compared to C, HA exhibited an elevated heat shock response [HSP70 (+ 99%); HSP60 (+ 216%); HSP32 (+ 40%); all $p < 0.01$] and reduced inflammatory and apoptotic signaling [p-NF-κB:NF-κB (− 99%); p-JNK (− 49%); all $p < 0.01$]. When compared to C + LPS, HA + LPS also exhibited an elevated heat shock response [HSP70 (+ 68%); HSP60 (+ 32%); HSP32 (+ 38%); all $p < 0.01$]. However, inflammatory and apoptotic responses in HA + LPS were increased [p-IκBα:IκBα (+ 432%); p-NF-κB:NF-κB (+ 283%); caspase-8p18 (+ 53%); p-JNK (+ 41%); all $p < 0.05$]. This unanticipated finding may be due to increased TLR4-mediated signaling capacity in HA + LPS, as indicated by upregulation of TLR4 [(+ 24%); MyD88 (+ 308%); p-NIK (+ 199%); and p-IKKα/b (+ 81%); all $p < 0.05$]. Data suggest HA reduces inflammatory and apoptotic signaling in skeletal muscle cells that are maintained under basal conditions. However, HACT is selective and does not apply to TLR4 signaling in the present model.

Keywords Skeletal muscle · Hyperthermia · Inflammation · Apoptosis

Abbreviations

GSK-3α/β Glycogen synthase kinase 3 alpha/beta
 p-HSF-1 Phosphorylated heat shock factor 1
 HSF-1 Heat shock factor 1

HSP32 Heat shock protein 32
 HSP60 Heat shock protein 60
 HSP70 Heat shock protein 70
 IRAK-1 Interleukin 1 receptor associated kinase
 p-IκBα Phosphorylated inhibitor of kappa beta
 IκBα Inhibitor of kappa beta
 p-IKKα/β Phosphorylated inhibitor of kappa beta kinase
 p-JNK Phosphorylated c-Jun N-terminal kinase
 MyD88 Myeloid differentiation primary response protein 88
 p-NF-κB Phosphorylated nuclear factor kappa beta
 NF-κB Nuclear factor kappa beta
 p-NIK Phosphorylated nuclear factor kappa beta initiating kinase
 PGC-1α Peroxisome proliferator-activated receptor gamma
 PPARs Peroxisome proliferator-activated receptors
 TLR4 Toll-like receptor 4

NEW AND NOTEWORTHY

When provided alone, HA confers diminished inflammatory and apoptotic signaling in C2C12 myotubes. These responses are in-line with the traditional concept of thermotolerance. However, they do not offer protection against subsequent LPS challenge. Instead, cells appear more prone to inflammation and apoptosis, an unanticipated outcome we show may be attributable to an increased TLR4-mediated signaling capacity.

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TNF- α Tumor necrosis factor alpha
IL-6 Interleukin 6

Introduction

The pathogenesis of exertional heat stroke (EHS) is driven by excessive hyperthermia that leads to the translocation of lipopolysaccharide (LPS) from the gastrointestinal tract into circulation (Kuennen et al. 2011; Leon and Bouchama 2015). Skeletal muscle, which is one of the largest and most metabolically active organs in the human body during exertional heat stress (Pedersen and Febbraio 2012), also expresses abundant toll-like receptor 4 (TLR4) (Frost et al. 2002). Circulating LPS binds to TLR4 on skeletal muscle, resulting in transcription of pro-inflammatory genes (Frisard et al. 2010) and secretion of pro-inflammatory cytokines into circulation (Eisele et al. 2013, 2015). LPS-mediated activation of skeletal muscle also contributes to a systemic inflammatory response syndrome in animal models of EHS (Frost et al. 2002; Horowitz 2016; Leon and Bouchama 2015; Lim and Mackinnon 2006; Welc et al. 2013) and in human patients that have been hospitalized for EHS (Huisse et al. 2008).

In contrast to the above, repeated exposure to modest hyperthermia promotes favorable adaptations in skeletal muscle. For example, Yamaguchi et al. (2010) reported 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 shift in myotube “fiber-type” that the authors attributed to activation of PGC-1 α (Yamaguchi et al. 2010). A recent study by our group indicates that 6 days of 2 h/day hyperthermia exposure (40 °C) increases AMPK, SIRT-1, and PGC-1 α levels in differentiated C2C12 myotubes (Patton et al. 2018). These changes promoted mitochondrial biogenesis that led to improved functional metabolic outcomes when myotubes that received hyperthermia treatment (HA) were subsequently challenged with LPS. Specifically, we noted that HA myotubes exhibited a reduced suppression of peak respiratory capacity in response to LPS challenge and an overall lower reliance on anaerobic glycolysis to maintain energy status (Patton et al. 2018). These findings are important because LPS compromises aerobic metabolism in skeletal muscle cells (Frisard et al. 2010; Park and Jeoung 2016). We referred to the metabolic improvements in that study as “metabolic cross-tolerance” (Patton et al. 2018) and noted that our findings in skeletal muscle cells paralleled the “heat-acclimation mediated cross-tolerance” (HACT) that had been reported by another group in cardiomyocytes (Assayag et al. 2012).

We expect these improvements may be due, in part, to activation of the cytoprotective heat shock response (HSR)

that is characterized by increased phosphorylation of heat shock factor 1 (HSF-1) (Dokladny et al. 2006) and results in elevated heat shock protein (HSP) content (Akerfelt et al. 2010; Kuennen et al. 2011). Indeed, exogenous HSP70 (derived from bovine skeletal muscle) reduces mortality rates and protects against impaired coagulation and fibrinolytic responses in LPS-challenged rats (Kustanova et al. 2006). In addition, we expect that HSP60 and HSP32 (both of which assist with mitochondrial protein import and macromolecule assembly in the mitochondrial matrix under stress conditions) (Akerfelt et al. 2010) may also play a role in mitochondrial biogenesis (Deocaris et al. 2006) and contribute to increased stress tolerance following HA. Beyond its role in binding to the heat shock elements on HSP genes to promote HSP induction, HSF-1 also inhibits pro-inflammatory cytokine expression via binding to the TNF- α promoter (Singh et al. 2002) as well as through physical interactions with the nuclear factor of IL-6 (Xie et al. 2002). There is also evidence that HSF-1 inhibits pro-inflammatory cytokine expression through regulation of the phosphorylation status of NF- κ B (Wirth et al. 2004) and downregulates apoptotic responses to proteotoxic stimuli by inhibiting phosphorylation of Jun N-terminal kinase (JNK) (Su et al. 2016). C2C12 myotubes that received 3 h/day hyperthermia exposure (39.5 °C) for 3 days prior to being exposed to a more severe thermal stress (4 h at 43 °C) were protected against apoptotic cellular death, as indicated by reductions in activation of caspase-3/7 cascades and cytochrome c release from mitochondria (Yu et al. 2016). In that study, protection against apoptosis may have been due to alterations in mitochondrial morphology, as myotubes that received the hyperthermia treatment exhibited “overly elongated mitochondrial networks” (Yu et al. 2016).

These improvements in the functionality of molecular cytoprotective networks suggest HA may also diminish inflammatory and apoptotic responses in skeletal muscle due to LPS exposure. Further research in this area could help unravel the mechanism(s) by which a standard HA protocol reduces the incidence of EHS in animals and humans. Interestingly, the transcriptional coactivator PGC-1 α has been shown to afford protection against hyperthermic stress in mice via cooperation with HSF-1 (Xu et al. 2016). In that study, PGC-1 α was shown to be a requisite for the development of heat resistance in response to thermal challenge, and both PGC-1 α and HSF-1 were shown to physically interact with the HSE element on the HSP70 promoter (Xu et al. 2016). This raises the question of whether increased PGC-1 α and HSF-1 in skeletal muscle following HA may help reduce the pathogenesis of EHS that is driven by the hyperthermia-mediated translocation of LPS from the gastrointestinal tract into circulation. Perhaps, in addition to protecting against metabolic compromise, the HA-mediated induction of PGC-1 α and HSF-1 in skeletal muscle might also contribute to a dampening of TLR4-mediated signaling cascades. In line with this

possibility, in prior work, we showed that a 7-day HA protocol activates the heat shock response and reduces circulating LPS concentrations in young, healthy males (Kuennen et al. 2013). The present study was undertaken to determine if the heat acclimation-mediated metabolic and cellular adaptations shown in our prior work afford protection against apoptosis and inflammation in response to moderate-dose (500 ng/ml) LPS challenge. We hypothesized that HA would reduce apoptotic and inflammatory signals in response to LPS challenge.

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 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 set point (40 °C). For that reason, each mild heat stress exposure was maintained for 127 min; afterwards, 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) and matches the protocol that was utilized in our prior report

(Patton et al. 2018). For reference, two other research groups have utilized repeated thermal exposure to stimulate acquisition of the heat acclimation (HA) phenotype in C2C12 myotubes. Both groups utilized a 5-day HA protocol that commenced on the first day of myotube differentiation (Guo et al. 2016; Liu and Brooks 2012).

Heat acclimation protocol with subsequent LPS challenge

The HA protocol that was described in the previous section was repeated on a separate set of C2C12 myotubes. Following 24 h recovery, myotubes that had either received 6-day HA or been maintained for the same duration under control conditions (37 °C) were challenged with 500 ng/ml LPS (from *Escherichia coli*) (sc-3535, Santa Cruz, CA, USA) for a period of 2 h. This dose was selected because it biases skeletal muscle towards glycolytic metabolism (Dugo et al. 2005) and has been used experimentally to study the impact of hyperthermia on pro-inflammatory cytokine responses in murine skeletal muscle (Welc et al. 2016). Following LPS exposure, culture media were exchanged, and cells were harvested 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 per sample) was separated by size by 8–10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred 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), imaged using the ChemiDoc Touch from Bio-Rad (Hercules, CA), and quantified using Image Lab from Bio-Rad (Hercules, CA). Protein expression was normalized to a representative β-actin, and protein activation/inactivation was normalized to either total protein or representative β-actin. Data presented in this

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

Primary antibody name	SCBT antibody identifier	SCBT number
β -Actin	β -Actin (C4):	sc-47778
Caspase-8p18	Caspase-8p18 (E-8)	sc-166320
p-Heat shock factor 1	p-HSF-1 (Ser 230)-R	sc-30443-R
Heat shock factor 1	HSF-1 (E-4)	sc-17757
Heat shock protein 32	HO1 (H-105)	sc-10789
Heat shock protein 60	HSP60 (N-20)	sc-1052
Heat shock protein 70	HSP70/HSC70 (W27)	sc-24
Interleukin 1 receptor associated kinase	IRAK-1 (H-273)	sc-7883
p-Inhibitor of kappa beta	p-I κ B- α (B-9)	sc-8404
Inhibitor of kappa beta	I κ B- α (H-4)	sc-1643
p-Inhibitor of kappa beta kinase alpha	p-IKK α/β (Ser 180/Ser 1818)-R	sc-23470-R
p-Jun N-terminal kinase	p-JNK (G-7)	sc-6254
Myeloid differentiation protein 88	MyD88 (E-11)	sc-74532
p-Nuclear factor kappa beta	p-NF κ B p65 (Ser 276)	sc-101749
Nuclear factor kappa beta	NF κ B p65 (C-20)	sc-372
p-Nuclear factor kappa beta initiating kinase	p-NIK (Thr 559)	sc-12957
Nuclear factor kappa beta initiating kinase	NIK (A-12)	sc-8417
Toll like receptor 4	TLR4 (25)	sc-293072

manuscript are meant to accompany a recent report by our group in this journal which described the effect of HA on PPAR signaling, mitochondrial biogenesis, and regulators of macromolecule synthesis (both with and without LPS stimulation) (Patton et al. 2018). To maintain consistency between the two studies, the same sets of cell lysates were analyzed and reported on in both manuscripts. Each of the blot images and bar graphs presented in this manuscript is representative of the mean $\pm$ SEM for three independent wells that received the same treatment.

Myotube viability and proliferation capacity

Cells were treated with HA or maintained under control conditions and one half of the cells in HA and control received subsequent LPS challenge (as described above). Following treatment, differentiation media were replaced with differentiation media containing 10% WST (*v/v*). Myotube viability and proliferation capacity were measured with a kit from Cayman Chemical (Item 10008883, Ann Arbor, MI, USA) and colorimetric signal intensity was measured at 450 nm temporally for 200 min.

Statistical analyses

All data are presented as group mean $\pm$ standard error of the mean. Protein content was analyzed by Student's *t* test. Cell viability was analyzed by two-way ANOVA with Bonferroni's correction for pairwise 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

Heat shock response The 6-day HA protocol increased HSF-1 phosphorylation ($+141\% \pm 31\%$; $p = 0.04$), whereas total HSF-1 content was unchanged ($p = 0.09$). The ratio of phosphorylated HSF-1 to total HSF-1 content was increased ($+248\% \pm 62\%$; $p = 0.01$). The 6-day HA protocol also increased cellular HSP content [HSP70 ($+99\% \pm 7\%$; $p < 0.01$), HSP60 ($+216\% \pm 38\%$; $p < 0.01$), HSP32 ($+40\% \pm 10\%$; $p = 0.02$)] (Fig. 1a).

Inflammation and apoptosis The 6-day HA protocol reduced I κ B phosphorylation ($-37\% \pm 6\%$; $p < 0.01$) and total I κ B content ($-43\% \pm 4\%$; $p = 0.01$). There was no difference in the ratio of p-I κ B/p-I κ B ($p = 0.31$). However, p-NF- κ B was reduced ($-91\% \pm 5\%$; $p < 0.01$) and total NF- κ B content was elevated ($+34\% \pm 3\%$; $p < 0.01$). As such, the ratio of p-NF- κ B/NF- κ B was reduced ($-99\% \pm 4\%$; $p < 0.01$), suggesting that HA reduced cellular drive towards inflammation (Fig. 1b).

We also measured cellular content of phosphorylated JNK and caspase-8p18 because both are involved in programmed cell death induced by the Fas ligand as well as other apoptotic stimuli. Phosphorylated JNK was reduced following the 6-day

Fig. 1 Heat acclimation activates the heat shock response and reduces cellular markers of inflammation and apoptosis. **a** Phosphorylated heat shock factor 1 (p-HSF-1) protein expression normalized to total HSF-1 and protein expression of heat shock protein 70 (HSP70), heat shock protein 60 (HSP60), and heat shock protein 32 (HSP32) in C2C12 myotubes following either 6-day hyperthermia protocol (40 °C; $n = 3$) or cellular maintenance under control conditions (37 °C; $n = 3$). **b** Phosphorylated inhibitor of kappa beta (p-Iκβ) and total Iκβ protein expression, phosphorylated nuclear factor kappa beta (p-NF-κB) protein

expression normalized to total NF-κB in C2C12 myotubes following either 6-day hyperthermia protocol (40 °C; $n = 3$) or cellular maintenance under control conditions (37 °C; $n = 3$). **c** Phosphorylated c-Jun N-terminal kinase (p-JNK) and caspase-8p18 protein expression in C2C12 myotubes following either 6-day hyperthermia protocol (40 °C; $n = 3$) or cellular maintenance under control conditions (37 °C; $n = 3$). Unless otherwise indicated, target protein expression was normalized to β-actin as loading control. The asterisk indicates $p < 0.05$ compared with normothermic (37 °C) control

HA protocol ($-49\% \pm 4\%$; $p < 0.01$), but we did not detect any change in caspase-8p18 ($p = 0.46$). These data are inconclusive but do suggest that the 6-day HA protocol may have reduced apoptotic responses in C2C12 myotubes (Fig. 1c).

Heat acclimation with subsequent LPS challenge

When the results of the present study are combined with our prior report that utilized the same in vitro experimental design (Patton et al. 2018), it appears C2C12 myotubes respond to a 6-day HA protocol by making the following cellular modifications: (1) increased stimulators of mitochondrial biogenesis and elevated mitochondrial content; (2) increased glycogen biosynthetic enzymes and reduced regulators of lipid biosynthesis; (3) activation of the heat shock response; (4) reduced drive towards pro-inflammatory signaling; and (5) possible reductions in apoptosis. Given that LPS increases inflammatory/apoptotic signaling and biases C2C12 myotubes towards anaerobic (glycolytic) metabolism (Frost et al. 2002), we next wanted to determine if myotube tolerance of a moderate dose (500 ng/ml) LPS challenge was improved following the 6-day HA protocol.

Heat shock response Similar to what was reported under non-LPS stimulated conditions, we noted an increase in HSP [HSP70 ($+68\% \pm 3\%$; $p < 0.01$), HSP60 ($+32\% \pm 3\%$; $p < 0.01$), HSP32 ($+38\% \pm 14\%$; $p = 0.03$)] in cells that received

HA prior to the LPS challenge (Fig. 2a). However, phosphorylation of HSF-1 was reduced in cells that had been previously heat stressed ($-33\% \pm 4\%$; $p = 0.02$). Although an inadequate heat shock response to moderate-dose LPS challenge is not desirable, we acknowledge that further activation of the heat shock response may have not have been necessary because HSF-1 and HSP contents had already been elevated by the HA protocol.

Inflammation and apoptosis Inflammatory and apoptotic signaling were both depressed following the 6-day HA protocol. For that reason, we anticipated that HA would also diminish inflammatory and apoptotic signaling responses to a subsequent LPS challenge. However, we were surprised to find that, instead, these signals were elevated. Specifically, p-IκB was increased ($+245\% \pm 45\%$; $p < 0.01$), whereas total IκB was depressed ($-31\% \pm 6\%$; $p < 0.01$) (Fig. 2b). The ratio of p-IκB to total IκB content was significantly increased in HA + LPS ($+432\% \pm 77\%$; $p < 0.01$), suggesting an increase in inflammation. Likewise, p-NF-κB was elevated in HA + LPS ($+81\% \pm 33\%$; $p = 0.04$) and total NF-κB was reduced ($-49\% \pm 12\%$; $p < 0.01$). The ratio of p-NF-κB to total NF-κB content was also elevated ($+283\% \pm 127\%$; $p < 0.05$) in HA + LPS. These changes in inflammatory status may have also increased cellular drive towards apoptosis, as phosphorylation of JNK was not suppressed in HA + LPS ($p = 0.11$) and caspase-8p18 content was increased ($+53\% \pm 21\%$; $p = 0.04$) (Fig. 2c).

Fig. 2 Heat acclimated myotubes do not activate a sufficient heat shock response to LPS challenge and exhibit elevated cellular markers of inflammation and apoptosis. **a** Phosphorylated heat shock factor 1 (p-HSF-1) protein expression normalized to total HSF-1 and protein expression of heat shock protein 70 (HSP70), heat shock protein 60 (HSP60), and heat shock protein 32 (HSP32) following 500 ng/ml LPS challenge for 2 h in C2C12 myotubes that had previously received either the 6-day hyperthermia protocol (40 °C; $n = 3$) or been maintained for the same duration under control conditions (37 °C; $n = 3$). **b** Phosphorylated inhibitor of kappa beta (p-Iκβ) and total Iκβ protein expression, phosphorylated nuclear factor kappa beta (p-NF-κB) protein expression

normalized to total NF-κB following 500 ng/ml LPS challenge for 2 h in C2C12 myotubes that had previously received either the 6-day hyperthermia protocol (40 °C; $n = 3$) or been maintained for the same duration under control conditions (37 °C; $n = 3$). **c** Phosphorylated c-Jun N-terminal kinase (p-JNK) and caspase-8p18 protein expression following 500 ng/ml LPS challenge for 2 h in C2C12 myotubes that had previously received either the 6-day hyperthermia protocol (40 °C; $n = 3$) or been maintained for the same duration under control conditions (37 °C; $n = 3$). Unless otherwise indicated, target protein expression was normalized to β-actin as loading control. The asterisk indicates $p < 0.05$ compared with normothermic (37 °C) control

Examining the discrepancy between HA alone and HA with subsequent LPS challenge

Data suggest that LPS tolerance was worse in myotubes that received HA, as indicated by elevations in inflammatory and apoptotic signaling. This was unexpected because following HA alone, myotubes exhibited elevated mitochondrial content (reported previously (Patton et al. 2018)), activation of the heat shock response, and reduced levels of inflammatory and apoptotic signaling proteins. To assess the functional significance of these changes in protein content, we performed a standard WST assay to examine cell viability and proliferation. As shown (Fig. 3a), WST values in HA exceeded those in control from 90 to 200 min of measurement. Interestingly, WST values in HA also exceeded those in the control + LPS challenge condition from 120 to 200 min and the HA + LPS challenge condition from 160 to 200 min (all $p < 0.05$). Area under the curve (AUC) was also calculated for WST data. As shown (Fig. 3b), the AUC for WST was $26.9 \pm 4.6\%$ higher in HA than control and $19.7 \pm 7.6\%$ higher in HA + LPS than control (all $p < 0.05$).

Because myotube viability was not reduced in HA + LPS, we next questioned whether this increase in inflammatory and apoptotic signaling may be due to HA altering cellular capacity to respond to LPS challenge. To answer this question, we

first blotted for TLR4 as well as myeloid differentiation primary response protein 88 (MyD88), the cytoplasmic adapter protein that links TLR4 to downstream activation of interleukin receptor kinase 1 (IRAK-1) and subsequent activation of the canonical NF-κB p65-mediated pro-inflammatory cascade. Elevated inflammatory capacity could also be due to the non-canonical NF-κB pathway that is coordinated by NF-κB inducing kinase (NIK) and IκK α/β. Specifically, NIK mediates the processing of the NF-κB precursor protein p100 into the mature p52 subunit in an IκK1/IκKα manner. In blotting for these proteins, we noted myotubes that received HA prior to LPS challenge exhibited increased levels of TLR4 ($+24\% \pm 4\%$; $p = 0.03$), MyD88 ($+308\% \pm 41\%$; $p < 0.01$), p-NIK ($+199\% \pm 71\%$; $p = 0.02$), and p-IκK α/β ($+88\% \pm 35\%$; $p = 0.05$) (Fig. 4a). IRAK-1 was not elevated in HA + LPS ($p = 0.45$), suggesting that non-canonical NF-κB signaling may be foundational to the cellular HA response and should be further investigated.

Because these findings appeared to suggest that a prior hyperthermic stimulus sensitizes (not protects) myotubes to subsequent LPS exposure, we wanted to clarify whether alterations in TLR4 and MyD88 were an acute response to the 2-h LPS challenge that followed HA, or if perhaps they had been elevated in response to the chronic HA stimulus but had not been detected because no ligand (LPS) had been added to

Fig. 3 Heat acclimation increases cellular viability and proliferation capacity. **a** Cellular viability and proliferation capacity following exposure to 6-day hyperthermia protocol [HA; $n = 6$] or maintenance under control conditions [C; $n = 6$], where following a 24-h recovery period one half of myotubes in each condition received 500 ng/ml LPS challenge for 2 h [HA + LPS ($n = 6$) and C + LPS ($n = 6$), respectively].

prime the system. In blotting for TLR4 and MyD88 in non-LPS-challenged samples, we noted that HA contributed to an elevation in TLR4 ($+32\% \pm 5\%$; $p < 0.01$) and also in MyD88 ($+47\% \pm 14\%$; $p = 0.02$) (Fig. 4b). We also blotted for IRAK-1, p-NIK, and p-I κ K α/β in our non-LPS-challenged samples but these signals were not detectable in either C or HA (data not shown). The lack of upregulation of these proteins in the absence of LPS challenge indicates that HA alone did not increase inflammation. However, elevations in TLR4 and MyD88 following HA confer an increased inflammatory signaling capacity, thereby increasing myotube responsiveness to subsequent LPS challenge and possibly contributing to elevations in apoptosis.

Discussion

Our data demonstrate that, by itself, the 6-day HA protocol activates the heat shock response and reduces inflammatory (and possibly apoptotic) signaling in C2C12 myotubes. However, contrary to our expectation, these changes do not afford myotubes protection against subsequent LPS challenge. Instead, they appear to exacerbate LPS-mediated cellular dysfunction. The mechanism behind this unanticipated outcome may be regulated (in part) by a hyperthermia-mediated increase in the protein content of TLR4, MyD88, NIK, and I κ K α/β that affords myotubes an increased capacity to respond to LPS challenge. Therefore, although the results of this study affirm prior reports of thermotolerance following repeated thermal exposure (Akerfelt et al. 2010; Kuennen et al. 2011; Li et al. 2000), they raise questions about the utility of this thermotolerance in protecting cells against subsequent LPS challenge.

Measurements began immediately afterwards and were continued for 200 min. One asterisk indicates $p < 0.05$ as compared to HA. **b** Area under the curve for cell viability and proliferation data following treatment of C2C12 myotubes as described above. One asterisk indicates difference between heat conditions; $p < 0.05$. Two asterisks indicate difference within conditions; $p < 0.05$.

Inflammation C2C12 myotubes that overexpress PGC-1 α exhibit reduced NF- κ B activity and suppressed pro-inflammatory gene expression in response to treatment with TNF α or LPS (Eisele et al. 2013). In our prior report, we noted that the same 6-day HA protocol that was utilized here elevated the PGC-1 α content of C2C12 myotubes (Patton et al. 2018). In the present study, the cytoprotective heat shock response was shown to be active following the 6-day HA. Myotube inflammatory responses were also shown to be reduced, as indicated by the reduced phosphorylation of NF- κ B. The increase in total NF- κ B content could be indicative of changes in myotube proliferation, as there is evidence that IKK/NF- κ B regulates myogenesis in skeletal muscle via a signaling switch that inhibits differentiation and promotes mitochondrial biogenesis (Bakkar et al. 2008). Given these observations, we anticipated that HA would reduce inflammatory and apoptotic responses to LPS challenge. In fact, the opposite finding was shown. Perhaps, this was due to our use of differentiated myotubes in these experiments that are more analogous to the in vivo condition. For reference, acute inflammation due to TNF α or LPS administration was not reduced by PGC-1 α overexpression in skeletal muscle fibers in vivo (Eisele et al. 2015). Increased GSK-3 content following HA (as reported in our prior manuscript (Patton et al. 2018)) may have contributed to the elevated inflammatory response to LPS exposure, as GSK-3 is known to be an important positive regulator of the inflammatory process. In fact, GSK-3 activity is required to achieve full production of the pro-inflammatory cytokines TNF α , IL-1 β , and IL-6 in peripheral blood mononuclear cells that are responding to TLR4 stimulation (Martin et al. 2005). Women with diet-controlled gestational diabetes exhibit elevated GSK-3 β activity in skeletal muscle tissues, as evidenced by decreased expression of GSK-3 β phosphorylated at serine 9 (Lappas 2014); whereas,

Fig. 4 The mechanism behind detrimental responses to LPS challenge in heat-acclimated myotubes may be linked to an elevated TLR4-mediated inflammatory signaling capacity. **a** Toll-like receptor 4 (TLR4), myeloid differentiation protein 88 (MyD88), interleukin 1 receptor associated kinase (IRAK-1), phosphorylated nuclear factor kappa beta initiating kinase (p-NIK), and phosphorylated inhibitor of kappa beta kinase alpha beta (p-IKK α/β) protein expression following 500 ng/ml LPS challenge for 2 h in C2C12 myotubes that had previously received

either the 6-day hyperthermia protocol (40 °C; $n = 3$) or been maintained for the same duration under control conditions (37 °C; $n = 3$). **b** TLR4 and MyD88 protein expression following either the 6-day hyperthermia protocol (40 °C; $n = 3$) or cellular maintenance under control conditions (37 °C; $n = 3$). Unless otherwise indicated, target protein expression was normalized to β -actin as loading control. The asterisk indicates $p < 0.05$ compared with normothermic (37 °C) control

addition of the GSK-3 inhibitor CHIR99021 to skeletal muscle and omental fat tissue explants from these women prior to LPS exposure reduces gene expression and secretion of pro-inflammatory cytokines (TNF- α , IL-1 β , and IL-6), pro-inflammatory chemokines (IL-8 and MCP-1), and adhesion molecules (ICAM-1 and VCAM-1) (Lappas 2014). In other studies, GSK-3 inhibition reduces the transcriptional activity of NF- κ B (Hoeftlich et al. 2000), conferring suppression of pro-inflammatory responses and providing reduced organ injury in rodent models of endotoxin shock (Dugo et al. 2005).

To date, research within the heat shock community has primarily focused on the TLR4-mediated induction of pro-inflammatory genes via the canonical NF- κ B p65 pathway (Liu et al. 2016; Ohno et al. 2011; Welc et al. 2013). To our knowledge, we are the first to examine the role of the non-canonical NF- κ B pathway in the establishment of HA, of which NF- κ B initiating kinase (NIK) and IKK α act as the primary signaling components. NIK is a MAP kinase kinase (MAP3K) family member and IKK α is the downstream kinase that triggers the phosphorylation-induced

processing of p100, resulting in the rapid nuclear translocation of the NF- κ B heterodimer p65/p50. De novo synthesis and accumulation of NIK are a requisite for induction of non-canonical NF- κ B signaling because NIK levels are negligible in unstimulated cells (Sun 2011). Our findings support this, as NIK was undetectable in control and HA cells prior to LPS challenge in the present study. Although LPS binding to TLR4 is recognized to play a role in non-canonical NF- κ B signaling, LPS's ability to induce p100 processing is weaker than some of the other, more recognized non-canonical NF- κ B signaling components. We should also point out that IKK α (the downstream kinase of NIK) phosphorylates NIK, triggering its proteolysis by way of a negative-feedback mechanism (Sun 2011). As such, it is possible that elevated pNIK and pIKK α/β levels in HA + LPS could simply reflect an upregulation of normal housekeeping functions in order to maintain cellular quality control (Bakkar et al. 2012).

Heat shock response, inflammation, and apoptosis HSF-1 and cellular HSP content are both known to downregulate apoptotic and necrotic cellular pathways, whereas a lack of

HSF-1 phosphorylation and HSP induction in cells responding to stress exposure increases inflammatory and apoptotic signaling (Li et al. 2000; Liu et al. 2016; Takayama et al. 2003). In the present study we noted a ~1.4-fold increase in HSF-1 phosphorylation ($p < 0.05$) and a trend towards increased total HSF-1 content ($p = 0.09$) following the 6-day HA protocol. The ratio of phosphorylated HSF-1 to total HSF-1 content was also increased by ~2.5-fold ($p = 0.01$), indicating HA caused a robust activation of the heat shock response. The lack of a significant increase in total HSF-1 content is most likely an artifact of our experimental protocol. Although HA myotubes were allowed to recover at 37 °C for 24 h following their last thermal stress exposure, this may not have been sufficient time to provide for full recovery. In other words, repeated daily thermal stress exposure may have contributed to the phosphorylation of newly formed HSF-1, thereby obscuring any increase in total HSF-1 content. Alternatively, the lack of increase in total HSF-1 content could indicate that our 6-day HA protocol was akin to short-term heat acclimation (STHA), wherein changes in the expression of genes responsible for the heat shock response and anti-apoptotic networks were evident but tolerance to subsequent stress exposure was not yet elevated (Assayag et al. 2012). This is an important observation, as long-term (30 days) HA produces an apoptosis-resistant cardiac phenotype in mice that affords protection against ischemia-reperfusion injury, whereas STHA (2 days) increases apoptosis susceptibility in the same mouse cohort (Assayag et al. 2010).

The lack of further increase in HSF-1 phosphorylation in HA myotubes responding to LPS challenge in the present study suggests that some of this protection had been lost. As outlined earlier in the discussion, we previously reported that HA causes GSK-3 β upregulation in C2C12 myotubes (Patton et al. 2018) that we expect may have contributed to their increased inflammatory capacity in response to LPS challenge. It is noteworthy then that GSK-3 β upregulation suppresses HSF-1 activation in neuroblastoma cells (Bijur and Jope 2000) and downregulates the DNA-binding and transcriptional activities of HSF-1 in oocytes (Xavier et al. 2000). However, reduced phosphorylation of HSF-1 in response to LPS challenge may not have been a completely undesirable phenomenon. HSF-1-mediated suppression of JNK activity promotes maintenance of mTORC1 integrity and activity, resulting in an increased translational capacity that allowed for hypertrophy of cells, organs, and tissues (Su et al. 2016). It is expected that myotubes in the present study expended a significant amount of cellular resources to increase mitochondrial content and maintain proteostasis over the 6-day HA protocol. This speculation is supported by the elevated myotube proliferation capacity in the WST assay and by the elevated mitochondrial content and OCR that we reported in our prior work (Patton et al. 2018). As such, HA may have reduced myotube energy reserves to the extent that they

were unable to tolerate the elevated energy requirements that were associated with the subsequent LPS challenge (Frisard et al. 2010; Park and Jeoung 2016). In that instance, the lack of increase in HSF-1 phosphorylation in HA myotubes that were responding to LPS challenge and the resultant increase in cellular marker of apoptosis may have been an intentional cellular strategy, as this would reduce myotube competition for the available energy supply and allow for the recycling of multiple high-energy cellular components during the apoptotic process (Marton et al. 1997).

Limitations Myotubes in the present study received 6 days of 2 h daily HA (40 °C) or were maintained for the same duration under control conditions (37 °C). Differences in protein expression between control and HA myotubes were examined 24 h following the last thermal exposure. The HA + LPS experiment replicated this protocol, wherein 24 h following the last thermal exposure, control and HA myotubes were challenged with 500 ng/ml LPS for 2 h. Differences in cellular protein expression were measured immediately following the 2-h LPS challenge. This time course may not have allowed sufficient time to see the full extent of changes in protein synthesis caused by LPS exposure. However, in support of this time course, Sharif et al. 2007 reported that the NF- κ B content of macrophages is elevated at 30 min post LPS stimulation and peaks at 2 h (Sharif et al. 2007). Also, in that study, inflammatory protein expression (TNF α , IL-1 β) peaked at 1 h post LPS stimulation before returning towards baseline values at 4 h post (Sharif et al. 2007). Using a proteomics approach, another group reported that the synthesis and abundance of multiple proteins that participate in the TLR4 signaling pathway were elevated immediately following 2 h LPS stimulation (Eichelbaum and Krijgsveld 2014). Importantly, both of those reports were in mouse macrophages, whereas the present study examined mouse myotubes. However, we have no reason to suspect that the rates of protein synthesis and/or changes in protein abundance would differ between these experiments.

Conclusions Our prior work in exercising humans showed that completion of a standard HA protocol activates the heat shock response and leads to positive adaptations in protein management and reduced inflammatory cytokine production via inhibition of the NF- κ B pathway (Kuennen et al. 2011). We have also shown that HA stimulates mitochondrial biogenesis in C2C12 myotubes, which improves general metabolic functions and improves myotube capacity to maintain aerobic metabolism during subsequent LPS challenge (Patton et al. 2018). LPS impairs metabolic responses in skeletal muscle cells (Frisard et al. 2010; Park and Jeoung 2016) and causes increased inflammatory myokine secretion (Leon and Bouchama 2015; Pedersen and Febbraio 2012). We had anticipated that our in vitro HA protocol would reduce these

Fig. 5 Working hypotheses of the mechanisms governing detrimental responses to LPS challenge in heat-acclimated myotubes. (1) Heat acclimation (HA) increases heat shock protein (HSP) content in C2C12 myotubes. HSP complex with heat shock factor 1 (HSF-1), reducing phosphorylation of HSF-1 in response to LPS challenge. (2) HA increases protein content of TLR4, MyD88, NIK, and IKK. This “primes the pump,” affording myotubes an increased inflammatory signaling capacity in response to LPS challenge. (3) As reported previously, HA increases GSK-3 content in C2C12 myotubes (Patton et al. 2018). GSK-3 causes phosphorylation of JNK, activating apoptotic cascades. GSK-3 has also been shown to repress the heat shock response

at both the transcript (HSF-1) and protein (HSP) levels. In addition, there is evidence that MyD88 increases JNK phosphorylation and inflammation is known to reinforce apoptotic signaling cascades. (4) As reported previously, repeated thermal exposure stimulates mitochondrial biogenesis (Patton et al. 2018). This could contribute to a biosynthetically mediated energy deficit that is made worse by subsequent LPS challenge. (5) To overcome the energy shortfall, cells upregulate catabolic processes (glycolysis and lipolysis) and initiate apoptotic cascades. This short-term strategy may serve to reduce myotube number and thus overall competition for macronutrient availability

responses in C2C12 myotubes that received a subsequent LPS challenge. Importantly, we verified that HA contributed to a robust activation of the HSR (as indicated by increased serine

230 phosphorylation of HSF-1 and elevated protein content of HSP32, HSP60, and HSP70) in conjunction with reduced inflammatory signaling (as indicated by reduced content of

proteins along the NF- κ B pathway) and apoptosis (as indicated by reduced Thr 183/Tyr 185 phosphorylation of JNK).

However, contrary to our hypothesis, HA cells did not exhibit a downregulation of inflammatory and apoptotic signaling in response to subsequent LPS exposure. Instead, prior thermal stress appeared to exacerbate the LPS-mediated disruption in oxidative capacity, causing an elevation in both inflammatory and apoptotic signaling. Through follow-up experiments, we determined that this unanticipated finding was associated with upregulations of TLR4, MyD88, and NIK, each of which was also present following HA alone (i.e., prior to LPS exposure). Collectively, our prior study that utilized the same experimental model (Patton et al. 2018) and the present report illustrate that the metabolic benefits of repeated thermal exposure in C2C12 myotubes may be mitigated by the accompanying increase in TLR4-mediated inflammatory signaling capacity. Given the myriad of known ligands for TLR4 (Carter et al. 2016) that are present in the bloodstream, which is largely redistributed towards skeletal muscle during exertional heat stress (Amorim et al. 2015; Horowitz 2016), it appears that further research is warranted in this area. Attempts to improve upon the current in vitro experimental design should also be explored, possibly through the incorporation of electrical stimulation to simulate skeletal muscle contraction, or hypoxia to simulate the alterations in oxygen tension that occur during exertional heat stress. Our work demonstrating increased susceptibility to LPS-mediated inflammatory and apoptotic dysfunction in HA myotubes will help to inform future research. We have generated a summary figure (Fig. 5) that highlights our major study findings.

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Author contribution MGP was the lead author of the manuscript. MCS, LMG, and CMW assisted with experiments and manuscript preparation. TLG assisted with manuscript preparation. RAV designed and conducted experiments and assisted with manuscript preparation. MRK conducted and oversaw all experiments, designed experiments, oversaw manuscript writing, performed statistical analyses, and conceived the study. All authors read and approved the final manuscript.

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