HEAT ACCLIMATION MEDIATED CROSSTOLERANCE IN C2C12 MYOTUBES

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ABSTRACT

Heat acclimation (HA) may protect skeletal muscle against novel stress exposure. **Purpose**: This study investigated HA-mediated cross tolerance in C2C12 myotubes. **Methods**: Differentiated myotubes were heated (40º C) for 2h/d over 6d (HA) or maintained at (37º C) (Control [CN]). HA and CN myotubes were challenged with hypoxia (1% F_IO_2 [H]) or hypoxia + LPS (500 ng/ml [H+L]) for 2h. Cell lysates were collected at +0h and +12h following challenge. Protein markers of heat shock response (HSR), inflammation, and apoptosis were assessed with western blot. Data were analyzed with two-way ANOVA with Newman-Keuls post-hocs. **Results**: Phosphorylation of HSF-1 was increased at +0h in HA [+59%, $p = 0.03$], HA (H) [+62%, $p < 0.01$] and HA (H+L) [+51%, p = 0.03], but did not increase until +12h in CN (H) [+86%, $p < 0.01$] and CN (H+L) [+77%, $p = 0.01$]. Likewise, HSP70 did not increase until +12h in CN (H) [+158%, $p = 0.01$] and CN (H+L) [+153%, $p = 0.04$]. In contrast, TLR4 (+77%, $p = 0.01$) and NFκB (+117%, $p = 0.03$) were increased in CN (H+L) at +12h. SIRT-1 was reduced in CN (H) [-55%, $p = 0.03$] and C (H+L) [-70%, $p < 0.01$] at +0h. This may have contributed to increased phosphorylation of JNK at +12h in CN (H) [+75%, $p < 0.01$] and CN (H+L) [+55%, $p = 0.03$]. At +12h terminal effector caspase-3 also trended towards increase in CN (H) [+28%, $p = 0.07$] and increased in CN (H+L) [+74%, $p = 0.02$]. **Conclusions**: HA activates the HSR and elevates SIRT-1, conferring lower inflammatory and apoptotic drive. This HA-mediated cross tolerance is not evident until +12h, suggesting benefits of HA could be missed if an extended time course is not followed.

INTRODUCTION

- We have previously shown that a 6d heat acclimation (HA) protocol activates the heat shock response (HSR) and improves aerobic metabolism during subsequent stress exposure (2h challenge with 500ng/ml LPS) in C2C12 myotubes[1].
- Interestingly, HA myotubes also exhibited increased cellular content of multiple inflammatory and apoptotic indicators, suggesting not all adaptations in our HA model were desirable[2].
- All measurements in those studies[1,2] were taken immediately following LPS challenge. The present study was undertaken to determine if our outlook on inflammatory and apoptotic responses may have been different if we had allowed for additional recovery time (+12h) following stress exposure.
- In addition, we wanted to determine if our 6d *in vitro* HA model improves tolerance to subsequent hypoxia exposure, as suggested by *in vivo* cross tolerance work (in human peripheral blood mononuclear cells) performed by one of our group members[3,4].

METHODS

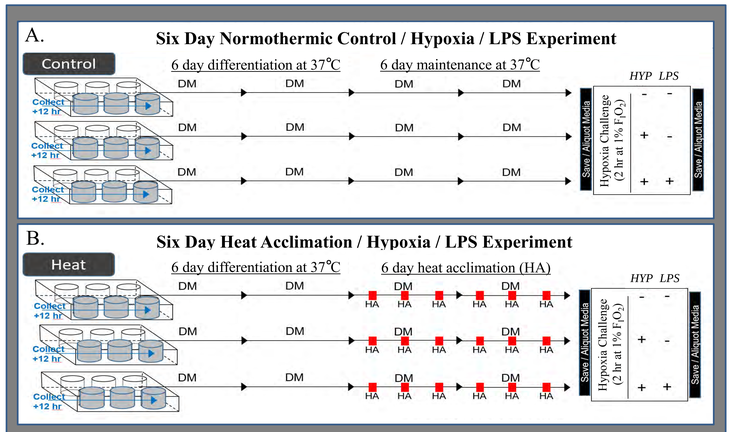

Figure 1: Schematic representation of experimental conditions. [A] Control myotubes were incubated at 37º C in differentiation media (DM) for 6 days (differentiation period). This was followed by a 6 d maintenance period, after which cells were challenged for 2 h with hypoxia (F_IO_2 = 1%), LPS (500ng/ml), or maintained under control conditions. Within each treatment (plate), one half of wells were collected immediately post challenge and the other at 12h post. [B] Procedures described in panel A were replicated with the addition of 2h daily heat treatments (40º C) to establish heat acclimation.

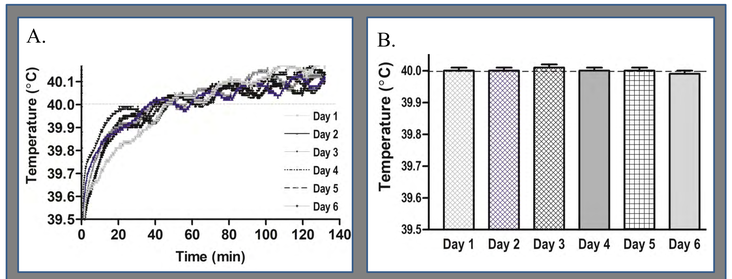

Figure 2. Graphical representation of incubator temperatures over the 6 day heat acclimation period. [A] To account for temperature changes upon incubator door opening, myotubes received 132 min of daily heat exposure. [B] There were no differences in incubator temperature across the 6d heat acclimation protocol. Data are Mean ±SEM for daily heat exposures.

RESULTS

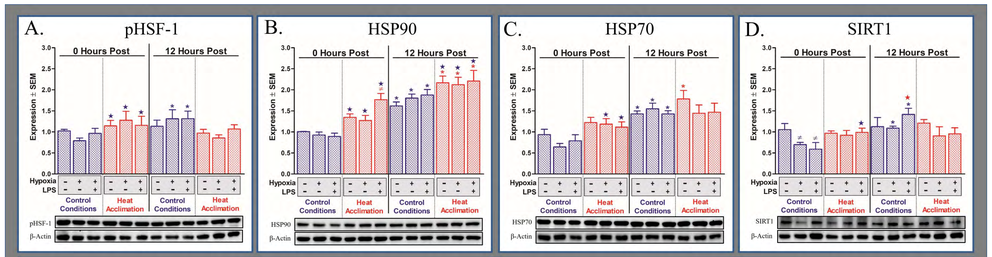

Figure 3. Heat acclimation activates the heat shock response (HSR) and increases SIRT1 expression in C2C12 myotubes. [A] Phosphorylated heat shock factor 1 (p-HSF-1), [B] heat shock protein 90 (HSP90), [C] heat shock protein 70 (HSP70), and [D] sirtuin 1 (SIRT1) following treatment of myotubes as described in methods. *indicates $p < 0.05$ compared with same treatment in opposite experimental condition. ♯ indicates $p < 0.05$ compared with other treatments in same experimental condition at same timepoint. *indicates $p < 0.05$ compared with same treatment in same experimental condition at earlier timepoint (0 hours post).

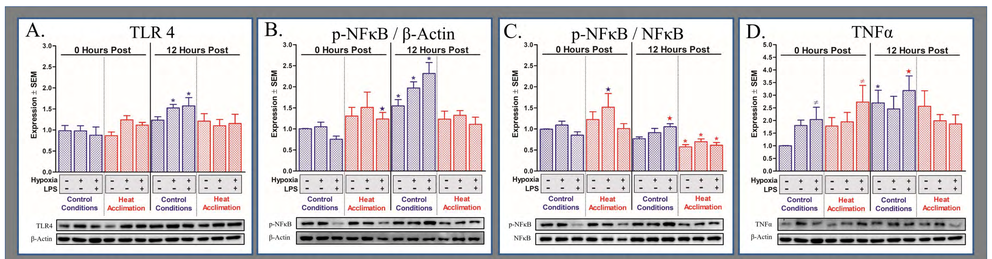

Figure 4. Heat acclimation elevates acute inflammatory response to hypoxia and hypoxia + LPS challenge but diminishes inflammatory response and TNFα production at 12 hours post challenge. [A] Toll like receptor 4 (TLR4), [B] phosphorylated nuclear factor-κB (p-NFκB) vs. β-actin as loading control, [C] p-NFκB vs. total NFκB content, and [D] tumor necrosis factor alpha (TNFα) following treatment of myotubes as described in methods. *indicates $p < 0.05$ compared with same treatment in opposite experimental condition. ♯ indicates $p < 0.05$ compared with other treatments in experimental condition at that timepoint. *indicates $p < 0.05$ compared with same treatment in same experimental condition at earlier timepoint (0 hours post).

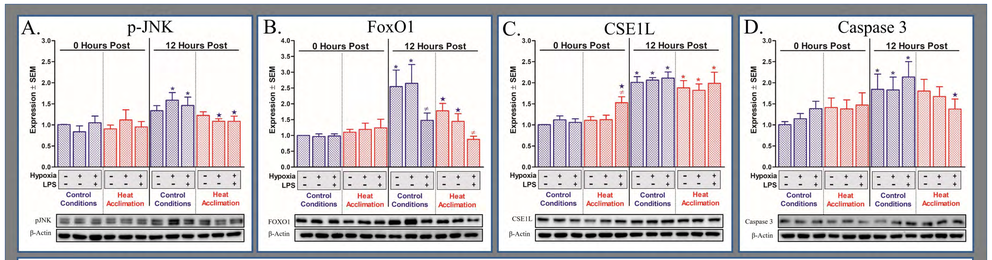

Figure 5. Heat acclimation reduces apoptotic response to hypoxia + LPS challenge. [A] Phosphorylated c-jun kinase (p-JNK), [B] forkhead box 1 (FoxO1), [C] cellular apoptosis susceptibility protein (CSE1L), and [D] caspase 3 following treatment of myotubes as described in methods. *indicates $p < 0.05$ compared with same treatment in opposite experimental condition. ♯ indicates $p < 0.05$ compared with other treatments in experimental condition at that timepoint. *indicates $p < 0.05$ compared with same treatment in same experimental condition at earlier timepoint (0 hour post).

CONCLUSIONS

- HA increased the HSP70 / HSP90 content of C2C12 myotubes. These proteins downregulate apoptotic signaling cascades and afford cytoprotection[5]. They also stabilize the confirmation of other molecules such as SIRT-1, which alleviates LPS-induced inflammation via reductions in TLR4 content and downregulation of NFκB signaling[6]. Control myotubes exhibited reduced SIRT-1 content following Hypoxia + LPS challenge. HA prevented this response.
- HA myotubes exhibited elevated inflammatory response immediately following Hypoxia and Hypoxia + LPS challenge. This finding confirms the earlier report by our group[2].
- However, we now suspect that NFκB signaling may be essential for resistance to apoptosis during HA[7], as cross-talk between NFκB and JNK has been shown to prevent sustained JNK activation[8]. In the present model this early activation of NFκB signaling in HA myotubes appears to be a beneficial response that prevented sustained induction of inflammatory and apoptotic signaling cascades (as evidenced by the reductions in p-NFκB, TNFα, p-JNK, FOXO1, and Caspase 3 in HA myotubes at 12h post Hypoxia + LPS challenge).

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