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Acute exercise in a hot environment increases heat shock protein 70 and peroxisome proliferator-activated receptor γ coactivator 1 α mRNA in Thoroughbred horse skeletal muscle

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Heat acclimatization or acclimation training in horses is practiced to reduce physiological strain and improve exercise performance in the heat, which can involve metabolic improvement in skeletal muscle. However, there is limited information concerning the acute signaling responses of equine skeletal muscle after exercise in a hot environment. The purpose of this study was to investigate the hypothesis that exercise in hot conditions induces greater changes in heat shock proteins and mitochondrial-related signaling in equine skeletal muscle compared with exercise in cool conditions. Fifteen trained Thoroughbred horses [4.6 ± 0.4 (mean ± SE) years old; 503 ± 14 kg] were assigned to perform a treadmill exercise test in cool conditions [COOL; Wet Bulb Globe Temperature (WBGT), 12.5°C; $n = 8$] or hot conditions (HOT; WBGT, 29.5°C; $n = 7$) consisting of walking at 1.7 m/s for 1 min, trotting at 4 m/s for 5 min, and cantering at 7 m/s for 2 min and at 90% of VO_{2max} for 2 min, followed by walking at 1.7 m/s for 20 min. Heart rate during exercise and plasma lactate concentration immediately after exercise were measured. Biopsy samples were obtained from the middle gluteal muscle before and at 4 h after exercise, and relative quantitative analysis of mRNA expression using real-time RT-PCR was performed. Data were analyzed with using mixed models. There were no significant differences between the two groups in peak heart rate (COOL, 213 ± 3 bpm; HOT, 214 ± 4 bpm; $p = 0.782$) and plasma lactate concentration (COOL, 13.1 ± 1.4 mmol/L; HOT, 17.5 ± 1.7 mmol/L; $p = 0.060$), while HSP-70 (COOL, 1.9-fold, $p = 0.207$; HOT, 2.4-fold, $p = 0.045$), PGC-1 α (COOL, 3.8-fold, $p = 0.424$; HOT, 8.4-fold, $p = 0.010$), HIF-1 α (COOL, 1.6-fold, $p = 0.315$; HOT, 2.2-fold, $p = 0.018$) and PDK4 (COOL, 7.6-fold, $p = 0.412$; HOT, 14.1-fold, $p = 0.047$) mRNA increased significantly only in HOT at 4 h after exercise. These data indicate that acute exercise in a hot environment facilitates protective response to heat stress (HSP-70), mitochondrial biogenesis (PGC-1 α and HIF-1 α) and fatty acid oxidation (PDK4).

KEYWORDS

heat stress, exercise, Thoroughbred horses, messenger RNA, skeletal muscle

1. Introduction

Heat acclimatization or acclimation training has generally been practiced in humans to reduce physiological strain, decrease aerobic metabolic rate, and improve exercise performance in the heat (1–5). Studies in humans and rodents have shown that these adaptations involve changes at the cellular level, including improved cytoprotection against heat stress, stimulated mitochondrial biogenesis, enhanced fatty acid oxidation, and angiogenesis promotion (6–9).

Heat shock proteins (HSPs) are classified as molecular chaperones that play a central role in the cellular stress response to a hot environment, and increased expression of HSPs has a protective function against cell injury associated with adverse stresses (10, 11). In addition, Henstridge et al. demonstrated that genetic overexpression of HSP-70 upregulates oxidative metabolism in rodent skeletal muscle and that HSP-70 may play an important role in the regulation of mitochondrial biogenesis (12). HSP-90, a member of the HSPs, also has specific characteristics and stimulates the activation of signaling transduction pathways distinct from those stimulated by HSP-70. Katschinski et al. demonstrated that the chaperone activity of HSP-90 is involved in hypoxic-inducible factor-1 α (HIF-1 α) stabilization *in vitro* (13). HIF-1 α regulates vascular endothelial growth factor (VEGF), which is implicated as important modulator of angiogenesis and facilitates migration. Both HSP-70 and HSP-90 augment proportionally increased cellular heat stress (14), in particular, HSP-70 gene expression increases synergistically when combined with exercise and hot conditions in rats (15). In addition, Morton et al. indicated that there is a sex specific HSP adaptation caused by estrogen in human skeletal muscle (16), and Gillum et al. have demonstrated that male increased HSP-72 more than female in peripheral blood mononuclear cells in response to running at 65% VO_{2peak} for 60 min in the heat (ambient temperature, 42°C; relative humidity, 20%) (17). In contrast, Mee et al. have demonstrated that there is no sex difference in leucocyte HSP72 mRNA after cycling at 65% VO_{2peak} for 90 min under a hot environment (ambient temperature, 40°C; relative humidity, 40%) in humans (18). However, there is limited information concerning the sex differences on HSPs in Thoroughbred horses.

Several studies have demonstrated that chronic heat stress can upregulate mitochondrial-related factors *in vitro* and *in vivo*, including humans and rodents. Repeated exposure to high temperature (40°C) can induce mitochondrial biogenesis and mitochondrial oxidative efficiency in C2C12 myotubes (19, 20). In addition, Maunder et al. demonstrated that endurance training under environmental heat stress (ambient temperature, 33°C; relative humidity, 80%) improves endurance performance and induces mitochondrial adaptation in humans (6). The increased energy demands associated with physiological strain activate the expression of the muscle peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1 α), a master regulator of mitochondrial biogenesis, gene expression, which can induce pyruvate dehydrogenase kinase 4 (PDK4) gene expression *via* estrogen-related receptor α (21). PDK4 facilitates the inactivation of pyruvate dehydrogenase complex, promoting an increase in mitochondrial fatty acid oxidation and a

concurrent decrease in glucose oxidation (22). In terms of acute exercise, Tamura et al. demonstrated that post-exercise in the chamber where ambient temperature regulated 40°C upregulates PGC-1 α expression in mouse skeletal muscle (7). In contrast, Heesch et al. demonstrated that acute exercise in a hot environment (ambient temperature, 33°C; relative humidity, 60%) blunts mitochondrial biogenesis related gene expression both immediately and at 3 h postexercise in humans (23). Therefore, the effect of acute exercise in a hot environment on mitochondrial adaptations is still controversial and needs further investigation.

Thoroughbreds have a high capacity for energy production and produce heat that elevates core temperature by approximately 1°C per minute during strenuous exercise (24). Furthermore, when these horses exercise in hot and humid environments (ambient temperature, 32–24°C; relative humidity, 80–85%), including racing or competition during daytime in summer, where heat dissipation is challenging, their core and muscle temperatures can rise to over 42°C during high-intensity exercise (25). Previously, we reported that exercise in an environment with an ambient temperature that exceeds body temperature causes a significant elevation of core body temperature and leads to decreased running economy (26). In addition, several other studies have demonstrated that exercise in a hot environment increases physiological strain and impairs exercise performance (25, 27). As a countermeasure to reduce these deleterious effects of heat stress, it is recommended that horses train in hot conditions similar to human athletes (28), and several studies have demonstrated that repeated exposure to heat stress can improve aerobic exercise performance and reduces metabolic rate in horses (29, 30). Therefore, we hypothesized that heat acclimatization or acclimation training would induce not only physiological adaptations but also cellular adaptations in skeletal muscle in horses. Several studies reported that the mRNA expression of heat shock proteins and mitochondria-associated proteins is increased after acute exercise in temperate conditions (31–33) and that there are no sex differences in the enzyme activities related to oxidative capacity in equine skeletal muscle (34). However, there is limited information concerning the differences in the acute signaling responses of equine skeletal muscle after exercise in a hot environment compared to exercise in a cool environment. Understanding these skeletal muscle responses can help construct a training strategy for races and competitions in a hot environment.

The purpose of this study was to investigate the hypothesis that exercise in hot conditions induces greater changes in heat shock proteins and mitochondrial-related signaling in equine skeletal muscle compared to exercise in cool conditions.

2. Materials and methods

2.1. Animals

Fifteen Thoroughbred horses [one male, seven geldings and seven females; age, 4.6 \pm 0.4 (mean \pm SE) years; body weight, 503 \pm 14 kg; maximal oxygen consumption (VO_{2max}), 174 \pm 7 (ml/

min kg)] were studied. Their characteristics are presented in Table 1. This study was conducted in Shimotsuke city (Japan) from February to March (average ambient temperature: 5–10°C) when the horses were not acclimatized to hot conditions. Horses had moderate-intensity training (1.7 m/s for 2 min, 4.0 m/s for 5 min, 7.0 m/s for 2 min, and 10.0 m/s for 2 min) on a treadmill (SÄTO AB, Knivsta, Sweden) at a 6% incline in cool conditions [ambient temperature, 20°C; relative humidity, 40%; Wet Bulb Globe Temperature (WBGT), 15°C; Temperature Humidity Index (THI), 62.8] 2 days/week for 4 weeks and walked for 1 h/day in a walker for 5 days/week and were kept in a 400 m² paddock for 6 h/day prior to the experiment. After training, the horses performed preliminary incremental exercise tests in cool conditions to determine their VO_{2max} . Following a warm up at 4 m/s for 3 min, the horses began exercising up a 6% incline for 2 min each at 1.7, 4, 6, 8, 10, 12 and 13 m/s until the horses could not maintain their position at the front of the treadmill with human encouragement.

2.2. Oxygen consumption

The procedure for measuring oxygen consumption has been described previously (35–37). Horses wore a 25 cm diameter open-flow mask on the treadmill, with a rheostat-controlled blower drawing air. Air flowed through 25 cm diameter tubing and across a pneumotachograph (LF-150B, Vise Medical, Chiba, Japan) connected to a differential pressure transducer (TF-5, Vise Medical, Chiba, Japan); this ensured that the bias flows during measurements were identical to those used during calibrations. Oxygen and CO₂ concentrations were measured by an O₂ and CO₂ analyzer (MG-360, Vise Medical, Chiba, Japan), and calibrations were performed to calculate rates of O₂ consumption and CO₂ production with electronic mass flow meters (CR-300, Kofloc, Kyoto, Japan) using the N₂-dilution/CO₂-addition mass-balance technique (38). Gas analyzer and mass flow meter outputs were recorded on personal computer and analyzed with commercial hardware and software (DI-720 and Windaq Pro+, DATAQ, Akron, OH). VO_2 was calculated for the final 30 s of each step at the incremental exercise test.

2.3. Experimental design

Horses were assigned to two groups, horses exercising in cool conditions (COOL, $n=8$) or horses exercising in hot conditions (HOT, $n=7$), to match their run time and VO_{2max} from the preliminary incremental exercise test. Each horse performed a treadmill exercise test consisting of walking at 1.7 m/s for 1 min, trotting at 4 m/s for 5 min, cantering at 7 m/s for 2 min, and eliciting 90% VO_{2max} (10.4 ± 0.2 m/s) for 2 min, followed by walking at 1.7 m/s

for 20 min in either cool conditions (COOL group, ambient temperature, 18.1 ± 0.5°C; relative humidity, 28.0 ± 2.5%; WBGT, 12.5 ± 0.5°C; THI, 61.2 ± 0.4) or hot conditions (HOT group, ambient temperature, 38.2 ± 0.4°C; relative humidity, 31.7 ± 0.4%; WBGT, 29.5 ± 0.3°C; THI, 77.3 ± 0.3). The room temperature was controlled using air conditioners (RAS-AP140DG4, Hitachi, Tokyo, Japan) and oil heaters (HPS360, Orion, Nagano, Japan). Ambient temperature, relative humidity, and WBGT were measured using a portable monitoring device (WBGT-213B, Kyoto Electronics Manufacturing, Kyoto, Japan).

2.4. Heart rate and plasma lactate concentration

A heart rate monitor (S810, Polar, Kempele, Finland) was attached around the thorax and mean heart rate was calculated for the final 30 s of each step of the exercise test. Venous blood was collected from the jugular vein via an 18-gauge needle into a heparin tube immediately after exercise. Blood samples were centrifuged (AX-511, Tomy industrial, Tokyo, Japan) at 1740 ×g for 10 min to measure plasma lactate concentration with a lactate analyzer (Biosen S-Line, EKF-diagnostic GmbH, Barleben, Germany).

2.5. Muscle biopsy

Before exercise (pre) and 4 h after exercise, muscle samples (~50 mg wet weight) were obtained from the same area (the two sampling points were approximately 2 cm apart) at the mid-section of the *gluteus medius* muscle and from the same depth (5 cm below the skin surface) by needle biopsy under local anesthesia (Lidocaine, Fujisawa pharmaceutical, Osaka, Japan). All muscle samples were immediately frozen by liquid nitrogen and stored at –80°C until analyzed.

2.6. RNA isolation and real time RT-PCR

The procedure for RT-PCR has been described previously (39). Total RNA was extracted from each muscle sample with a TRIZOL regimen (Molecular Probes, Breda, Netherlands). The purity and quantity of total RNA were determined by measuring the absorbance of aliquots at 260 and 280 nm. Total RNA was then treated for 30 min at 37°C with TURBO DNase (Ambion, Austin, TX, United States) to remove genomic DNA from samples. DNase-treated RNA (0.5 µg) was used to synthesize first-stand cDNA with an Exscript™ RT reagent Kit (Takara, Tokyo, Japan). Thereafter, the cDNA products were analyzed by real-time PCR using the SYBR Green PCR Master Mix protocol in a StepOne™ Real Time PCR System (Applied Biosystems Japan, Tokyo, Japan).

The amplification program included an initial denaturation step at 95°C for 10 min, 40 cycles of denaturation at 95°C for 30 s, and annealing/extension at 58°C for 1 min. The amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was estimated as an internal control (40). Each mRNA was normalized to GAPDH by subtracting the cycle threshold (Ct) value of GAPDH from the Ct value of the gene target [ΔCt (target)]. The relative

TABLE 1 Characteristics of fifteen horses used in the present study.

	Age (years)	Weight (kg)	VO_{2max} (ml/min kg)
Male ($n=1$)	4	513	164
Gelding ($n=7$)	5.1 ± 0.4	528 ± 24	174 ± 8
Female ($n=7$)	3.9 ± 0.6	477 ± 16	168 ± 5

expression of the target gene was calculated as the relative quantification value for the pre value. Following the relative expression, dissociation curve analysis detected no nonspecific amplification in cDNA samples.

The sequences of the specific primers used in this study are presented in Table 2. Each PCR primer was designed by Primer Express® software (Applied Biosystems Japan, Tokyo, Japan), and oligonucleotides were purchased from FASMAC (FASMAC, Kanagawa, Japan).

2.7. Statistical procedures

All data are presented as mean ± standard error (SE). Differences in heart rate and plasma lactate concentration between COOL and HOT during exercise were analyzed using Student's *t*-test. We analyzed mRNA expressions using mixed model with time, group and sex as fixed effects and individual horse as a random effect. When a significant effect or interaction was observed, Tukey's tests were used as *post hoc* tests. For all analyses, significance was set as $p \leq 0.05$. Statistical software (JMP 16.2.0, SAS Institute Inc., Cary, NC) was used for all data analyses.

3. Results

3.1. Heart rate and plasma lactate concentration

There were no differences between groups in peak heart rate during exercise (COOL, 213 ± 3 bpm; HOT, 214 ± 4 bpm; $p = 0.782$; Figure 1A). Plasma lactate concentration immediately after exercise in HOT tended to be higher than that in COOL, but the difference was not significant (COOL, 13.1 ± 1.4 mmol/L; HOT, 17.5 ± 1.7 mmol/L; $p = 0.060$; Figure 1B).

3.2. Gene expression

HSP-70 mRNA expression demonstrated a significant time effect (2.1-fold, $p = 0.003$), but not a group effect ($p = 0.433$) or interaction ($p = 0.462$, Figure 2A). At 4 h after exercise, HSP-70 mRNA increased significantly only in HOT compared to pre (2.4-fold, $p = 0.045$), but not in COOL (1.9-fold, $p = 0.207$). In contrast, HSP-90 mRNA did not change in either group at 4 h (COOL, 1.3-fold, $p = 0.189$; HOT, 1.4-fold, $p = 0.080$, Figure 2B), although a time effect was observed (1.4-fold, $p = 0.005$) similar to HSP-70. PGC-1 α mRNA demonstrated a significant time effect (6.0-fold, $p = 0.002$), but not a group effect ($p = 0.080$) or interaction ($p = 0.110$, Figure 3A). PGC-1 α mRNA levels increased in HOT (8.4-fold, $p = 0.010$) at 4 h compared to pre, but not in COOL (3.8-fold, $p = 0.424$). PDK4 mRNA expression demonstrated a significant time effect (10.6-fold, $p = 0.006$), but not a group effect ($p = 0.270$) or interaction ($p = 0.300$, Figure 3B). In addition, an increase in PDK4 mRNA was observed only in HOT at 4 h compared to pre (14.1-fold, $p = 0.047$), but not in COOL (7.6-fold, $p = 0.412$). SDHa mRNA expression did not reveal a significant time effect (1.1-fold, $p = 0.235$), group effect ($p = 0.559$), or interaction ($p = 0.503$, Figure 3C). HIF-1 α mRNA expression demonstrated a significant time effect (1.9-fold, $p = 0.002$), but not a group effect ($p = 0.274$) or interaction ($p = 0.212$, Figure 4A). A significant increase in HIF-1 α mRNA levels was observed only in HOT at 4 h compared to pre (2.2-fold, $p = 0.018$), but not in COOL (1.6-fold, $p = 0.315$). VEGF, ANGPT1 and CD31 mRNA expressions demonstrated significant time effects (2.4-fold, $p = 0.006$, 1.5-fold, $p = 0.019$, 1.7-fold, $p = 0.020$, respectively; Figures 4B–D), but did not change at 4 h compared to pre in either group (VEGF: COOL, 2.3-fold, $p = 0.293$; HOT, 2.5-fold, $p = 0.136$; ANGPT1: COOL, 1.7-fold, $p = 0.221$; HOT, 1.3-fold, $p = 0.901$; CD31: COOL, 1.7-fold, $p = 0.122$; HOT, 1.7-fold, $p = 0.153$). PFK mRNA did not show a significant time effect (1.1-fold, $p = 0.227$), group effect ($p = 0.434$), or interaction ($p = 0.185$, Figure 5A). MCT1 and MCT4 mRNA expression demonstrated a significant time effect (1.8-fold, $p = 0.022$; 1.3-fold, $p = 0.023$, respectively; Figures 5B,C) but did not

TABLE 2 Real-time reverse transcription (RT)-PCR primer sequences.

	Forward sequence	Reverse sequence
GAPDH	CAAGGCTGTGGCAAGGT	GGAAGCCATGCCGTGA
HSP-70	CATCCTGAACGTCACGGCCAC	CCTTCTTGTGTC(C/A/G)GCCTCG
HSP-90	CCCTGTTGTGGCGTGTGA	CGAGCACTACAGGGCAAGGT
HIF-1 α	AAGTGCAGACACGATTACAGTAT	GACGGTAGGAAGAGCAGGTTCTT
VEGF	CCCACTGCGGAGTTCAACAT	TTGGCTTTGGTGGGTTTGAT
ANGPT1	GCAAATGTGCCCTCATGCT	CAGATTGGATGGCCACAAG
PGC-1 α	TCCGTGTCACCACCCAAAT	TGAACGAGAGCGCATCCTT
PDK4	GCTGGTTTTGGTTATGGCTTGC	TCCACAGACTCAGAAGACAAAGCC
SDHa	AGGTTTGCTGATGGCAGTATAAGA	TGCATCGACTTCTGCATGCT
PFK	GGTGGCACAGTGATTGGAAGT	CGGAGTCGTCCCTCTCGTT
MCT1	GATTCTTGGCGGCTGCTTGTGAGG	TGCCAATCATGGTCAGAGCCGGA
MCT4	ATGGTGTCTGCGTCTTCTGCGGA	AGCGCCAAACCCAAAGCCGGTAA
CD31	GCAGATGATTCTGTGTTCCAA	TGGGAGCAGGGCAGGTT

GAPDH, glyceraldehydes-3-phosphate dehydrogenase; HSP-70, heat shock protein 70; HSP-90, heat shock protein 90; HIF-1 α , hypoxia inducible factor-1 α ; VEGF, vascular endothelial growth factor; ANGPT1, angiopoietin-1; PGC-1 α , peroxisome proliferator-activated receptor γ coactivator-1 α ; PDK4, pyruvate dehydrogenase kinase 4; SDHa, succinate dehydrogenase complex flavoprotein subunit A; PFK, phosphofructokinase; MCT1, monocarboxylate transporter 1; MCT4, monocarboxylate transporter 4; CD31, cluster of differentiation 31.

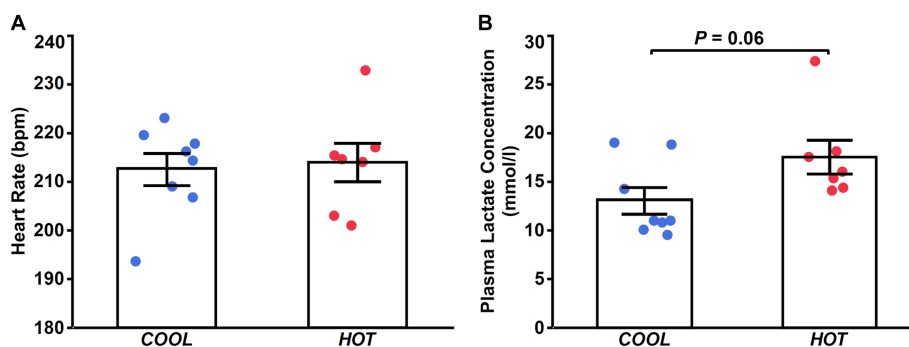


FIGURE 1

Heart rate (A) at the end of exercise and plasma lactate concentration immediately after exercise (B) in COOL (blue) and HOT (red).

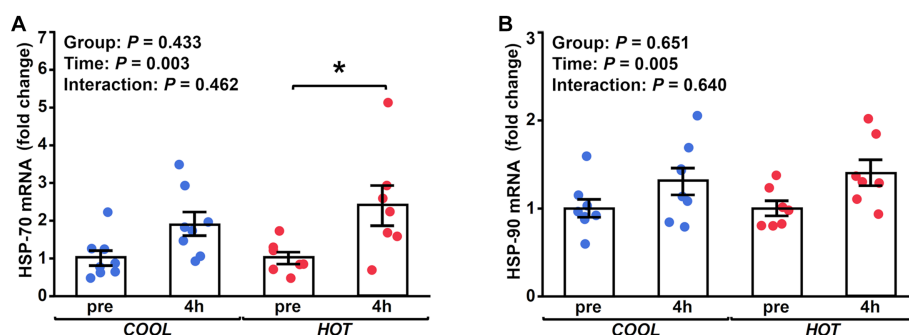


FIGURE 2

Fold changes in the mRNA expression of HSP-70 (A) and HSP-90 (B) at 4 h after exercise compared to before exercise (pre) in COOL (blue) and HOT (red). *Significant differences from pre ($p < 0.05$).

change in both groups at 4 h compared to pre (MCT1: COOL, 1.9-fold, $p = 0.067$; HOT, 1.6-fold, $p = 0.146$; MCT4: COOL, 1.3-fold, $p = 0.442$; HOT, 1.3-fold, $p = 0.767$). We did not observe any significant effects on sex in any variables (HSP-70, $p = 0.268$; HSP-90, $p = 0.184$; PGC-1 α , $p = 0.164$; PDK4, $p = 0.446$; SDHa, $p = 0.803$; HIF-1 α , $p = 0.461$; VEGF, $p = 0.424$; ANGPT1, $p = 0.245$; CD31, $p = 0.414$; PFK, $p = 0.358$; MCT1, $p = 0.374$; MCT4, $p = 0.720$).

4. Discussion

Our main finding in this study is that exercise in a hot environment upregulates the expression of HSP-70 mRNA and PGC-1 α mRNA in Thoroughbred horses. These results indicate that the combination of heat exposure and exercise facilitate the activation of heat stress responses and mitochondrial adaptations in Thoroughbred horses.

HSP-70 is classified as molecular chaperone that plays a central role in the cellular stress response to hot environments and has a protective function against cell injury associated with adverse stresses (41). Previous studies have shown that acute thermal stress with an increase in body temperature enhances HSP-70 mRNA expression in various species, including humans and rodents (7, 15, 42, 43). Heat shock factor 1, which is a direct transcriptional activator that induces heat shock gene transcription following heat stress, directly activates

the expression of PGC-1 α , a master regulator in mitochondrial biogenesis (44, 45). Although we did not measure body core temperature during exercise in this study, previous studies have demonstrated that pulmonary artery temperature during exercise under hyperthermic conditions (WBGT 28°C) is 1–2°C higher than that under cool conditions (WBGT 15°C) in horses (25–27). Therefore, it is reasonable to assume that core and muscle temperature in HOT was also higher than that in COOL, inducing sufficient thermal stress to upregulate the expression of these genes. Furthermore, the expression of PGC-1 α mRNA is enhanced in an intensity-dependent manner (46–48). In horses, several studies have demonstrated that VO_2 during submaximal exercise in hot conditions is higher compared to that at the same speed in cool conditions (26, 27), and increased relative workload may also contribute the upregulation of PGC-1 α mRNA. Therefore, additional increases in core and muscle temperatures with the combination of heat exposure and exercise enhance HSP-70 and PGC-1 α mRNA responses, which may be beneficial for cytoprotection and mitochondrial biogenesis.

While the expression of HSP-70 mRNA increased following exercise, no significant change was observed in HSP-90 mRNA. HSP-90, similar to HSP-70, plays a crucial role in cytoprotection and acts as biochemical sensors for various stressors, including heat shock and exercise (41). The extent of HSPs induction following a single bout of exercise depends on several factors, including exercise modality, duration, intensity, and training status of

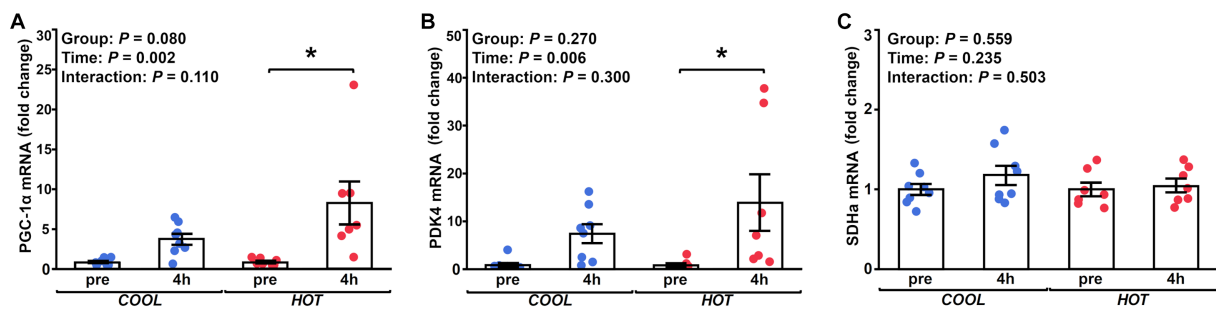


FIGURE 3

Fold changes in the mRNA expression of PGC-1 α (A), PDK4 (B), and SDHa (C) at 4h after exercise compared to before exercise (pre) in COOL (blue) and HOT (red). *Significant differences from pre ($p < 0.05$).

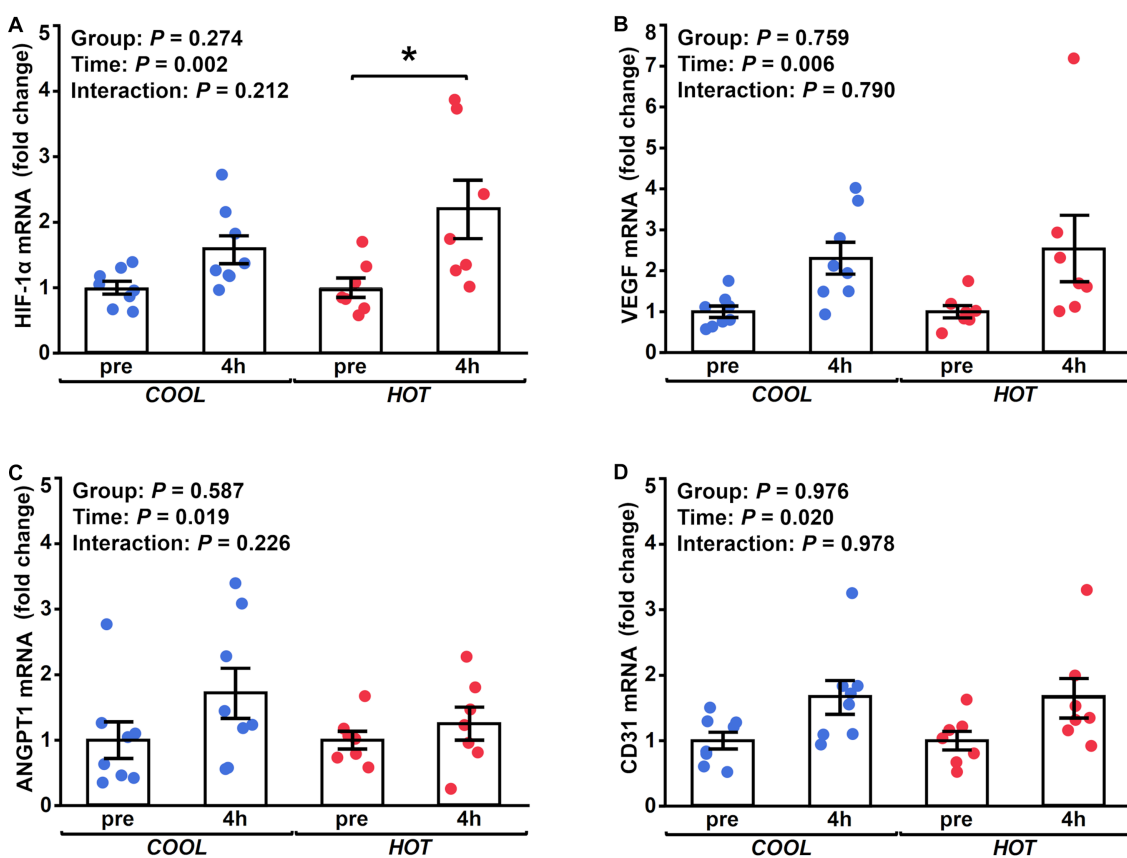


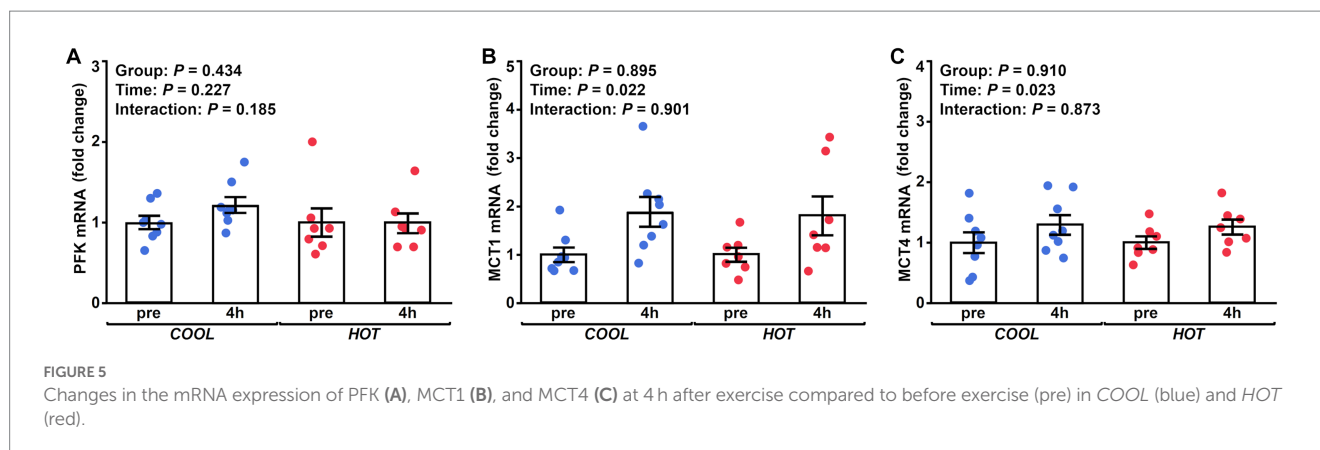
FIGURE 4

Changes in the mRNA expression of HIF-1 α (A), VEGF (B) and ANGPT1 (C), and CD31 (D) at 4h after exercise compared to before exercise (pre) in COOL (blue) and HOT (red). *Significant differences from pre ($p < 0.05$).

the subject (41, 49). Skidmore et al. demonstrated that a combination of exercise and a hot environment increases synergistic HSP-70 induction compared to either alone in rats (15). In addition, Cuthbert et al. demonstrated that HSP-70 mRNA increases 2- to 4-fold after moderate-intensity exercise (60% VO_{2peak}) in trained humans, while HSP-90 mRNA remains unchanged. Our findings are consistent with the results of Cuthbert et al. that indicate that the sensitivity response to stress differs depending of the type of HSP and that HSP-70 may be a more sensitive marker of heat stress compared with HSP-90 in trained Thoroughbred horses.

In the present study, we detected increased PDK4 mRNA coincident with increased PGC-1 α mRNA in Thoroughbred horses training in hot conditions (21, 22). Several studies have demonstrated that muscle glycogen utilization is reduced with heat acclimation (8, 50). Thus, the improved muscle energy metabolism with heat acclimation may involve a shift in energy fuel utilization from glucose oxidation to fatty acid oxidation due to the upregulation of PDK4.

In a hot environment, cutaneous blood flow increases through peripheral vasodilation for heat dissipation in horses and humans (51, 52). During prolonged low-to-moderate intensity exercise, muscle



blood flow is not reduced because an increase in cardiac output can compensate the increased skin blood flow (2, 53). However, González-Alonso and Calbet demonstrated that blood flow to exercising muscle is reduced during high-intensity exercise (cycling at 80% of peak power output) starting with high skin and core temperature in humans (54). Therefore, the blood flow demands from skin and muscle may exceed the cardiac capacity, and blood flow to skeletal muscle may have reduced during high-intensity exercise (90% $\text{VO}_{2\text{max}}$) in this study. HIF-1 α activates in response to hypoxia in various tissues, including skeletal muscle, and plays an integral role in regulating the various genes involved in the hypoxic response (55). Reduced blood flow to skeletal muscle during intense exercise in hot conditions may cause more severe hypoxia in skeletal muscle tissue. Therefore, exercise in a hot environment may result in cellular adaptation that counteracts the effects of reduced oxygen supply to cells by activating HIF-1 α . While we observed increased HIF-1 α mRNA, no changes were detected in VEGF, ANGPT1, and CD31 mRNA levels in each group. VEGF, ANGPT1, and CD31 are factors related to angiogenesis and are located downstream of HIF-1 α . In contrary to our findings in this study, Gustafsson et al. demonstrated that a single bout of cycling increased VEGF mRNA in human skeletal muscle and that this increase positively correlated with the change in HIF-1 α mRNA (56). Additionally, Gustafsson et al., also demonstrated a correlation between the increase in VEGF mRNA and plasma lactate concentration after exercise (56). In humans, exercise in a hot environment often enhances glycolysis in skeletal muscle, which may be probably due to reduced blood flow and oxygen delivery to working muscle (57). In this study, we observed no differences in the plasma lactate concentration between *HOT* and *COOL*. Therefore, the reduction in muscle blood flow and HIF-1 α induction may be insufficient to enhance angiogenesis in this study.

We observed no changes in PFK, MCT1, and MCT4 mRNA expressions in this study. PFK is a kinase enzyme that phosphorylates fructose 6-phosphate and is the main rate-controlling enzyme of glycolysis. MCT1 and 4 are key factors in mediating lactate transport. There were no differences in the plasma lactate concentration between *HOT* and *COOL*, which is reasonable in that gene expressions related to the glycolysis pathway and lactate transport were not enhanced in *HOT*. Previous studies in humans and horses have showed an augmented intramuscular carbohydrate utilization and an increased blood lactate concentration when comparing prolonged submaximal

exercise in a hot environment with that in a cool environment (1, 27, 58). In contrast, Maxwell et al. demonstrated no differences in the rate of muscle glycogenolysis and blood lactate accumulation during intermittent high-intensity exercise between hot and cool environments (59, 60). These results, including our results, indicate that the increased glycolysis and blood lactate accumulation may depend on several factors, including the duration and intensity of exercise, training status of subjects, environmental temperature, and the timing of sample collection.

We acknowledge there are limitations in this study. First, we obtained muscle samples only at 4h after exercise and did not observe time-course changes of gene expressions over time. Because the time-course changes of gene expressions vary with target markers (61), we could detect more mRNA changes if we collected muscle samples at several time points. Second, we only observed changes in mRNA expression following acute exercise. In humans, 10 days or longer heat acclimation are recommended to induce optimize adaptations (62). Some reports in horses have demonstrated that heat acclimation have beneficial effects on physiological responses similar to humans (29, 63). However, these studies did not evaluate cellular adaptations in skeletal muscle. Therefore, further studies are necessary to fully investigate the long-term effects of heat acclimatization or acclimation on skeletal muscle adaptations.

In summary, we have demonstrated that HSP-70, PGC-1 α , HIF-1 α , and PDK4 mRNA significantly increased after high-intensity exercise in hot conditions. These increases in gene expressions may facilitate a protective response to heat stress, mitochondrial biogenesis, and fatty acid oxidation. Therefore, our results suggest that heat acclimatization or acclimation for competitions and races in hot conditions is effective in improving aerobic energy metabolism, as well as reducing heat stress. Repeated exposure to heat stress, such as training in natural hot environment or in artificial heated environment, may be effective strategy for competitions and races held in hot environments.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by the Animal Welfare and Ethics Committee of the Japan Racing Association's Equine Research Institute (accession number 21-1).

Author contributions

YE, KM, YT, and HO contributed to the conception and design of the study. YE, KM, YT, TY, AK, TM, HM, and HO carried out the experiments. YE, KM, YT, TY, and HO performed data collection. YE, AK, TM, and HM analyzed the data. YE wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

YE, KM, YT, TY, and HO are employees of the Japan Racing Association.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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