

Transcriptional, but not post-translational control of PGC-1 α is blunted following exercise in a hot environment

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Running head: PGC-1 α response to exercise in a hot environment

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Abstract

Previous work has reported a reduced mRNA response related to mitochondrial biogenesis after exercise in a hot environment compared to room temperature conditions. The purpose of this study was to determine mitochondrial biogenesis-related mRNA expression, binding of transcription factors to the PGC-1 α promoter, and sub-cellular location of PGC-1 α protein in human skeletal muscle following exercise in a hot environment as compared to a room temperature environment. Recreationally trained males ($n = 11$, age: 24 ± 3 y, height: 178 ± 5 cm, weight: 80.3 ± 12.8 kg, % BF: $14.6 \pm 3.6\%$, VO_2 peak: 4.34 ± 0.84 L · min $^{-1}$) completed two trials in a temperature and humidity controlled environmental chamber. Each trial consisted of cycling in either a hot (H) or room temperature (C) environment (33^0 C and 20^0 C, respectively) for one hour at 60% of W_{max} followed by 3 h of supine recovery at room temperature. Muscle biopsies were taken from the *vastus lateralis* pre-, post-, and 3 h post-exercise for analysis of mitochondrial biogenesis-related mRNA expression, transcription factor binding to the promoter region of the PGC-1 α gene, and PGC-1 α protein subcellular location. PGC-1 α , ERR α , and VEGF mRNA increased from pre- to 3 h post-exercise in C ($p = 0.002$, $p = 0.011$, and $p < 0.001$, respectively). PGC-1 α , ERR α , NRF-1, GABPA, SIRT-1, and VEGF mRNA were all lower in H than C 3 h post-exercise (all $p < 0.05$). Binding of CREB ($p = 0.005$), MEF2 ($p = 0.047$), and FoxO1 ($p = 0.010$) to the promoter region of the PGC-1 α gene was lower in H than C, while ATF2 binding was not different between trials ($p = 0.124$). Nuclear PGC-1 α protein increased following exercise in both H and C ($p = 0.029$), but was not different between trials ($p = 0.602$). These data indicate that exercise in a hot environment blunts expression of mitochondrial biogenesis-related mRNA as compared to exercise in a room temperature environment due to decreased binding of CREB, MEF2, and FoxO1 to the PGC-1 α promoter, despite similar increases in nuclear PGC-1 α translocation.

Keywords: Mitochondrial biogenesis, skeletal muscle, heat stress, cycling

Introduction

Mitochondrial adaptation is important for both maintaining optimal health as well as improving athletic performance. Interventions to stimulate mitochondrial development are of interest as the dysfunction of mitochondria have been implicated in many conditions including diabetes (1-3), peripheral arterial disease (4), and aging (5, 6). The growing number of individuals with these mitochondrial related pathologies demonstrates the importance of understanding human mitochondrial biogenesis and possible interventions to stimulate mitochondrial biogenesis. Exercise training is one known intervention that leads to mitochondrial biogenesis in humans (7). While exercise alone is a valuable therapy to stimulate mitochondrial development, reduced capacity to perform adequate exercise in a sedentary or diseased population demonstrates the need to further enhance the effectiveness of a given exercise bout to stimulate mitochondrial development. Environmental temperature may play an important role in regulation of mitochondrial biogenesis. Mild heat stress has been shown to induce mitochondrial biogenesis in C2C12 myotubes (8). Alternatively, acute heat stress in yeast models has demonstrated a down-regulation in mitochondrial function (9), while chronic heat stress has been shown to inhibit mitochondrial development in chickens (10). Early markers of mitochondrial biogenesis appear to be blunted in humans following exercise in a hot environment (11). However, additional insight into this mechanism is necessary to better understand the impact of acute heat stress during exercise on mitochondrial biogenesis in a human model.

Normal function of the mitochondria is largely regulated by peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1 α), a transcriptional co-activator known as a master regulator of metabolism and mitochondrial biogenesis (12). PGC-1 α binds to and co-activates several transcription factors, including nuclear respiratory factor 1 (NRF-1), myocyte enhancer factor 2 (MEF2), estrogen-related receptor alpha (ERR α), GA binding protein transcription factor, alpha subunit 60kDa (GABPA, also known as NRF-2), and mitochondrial transcription factor A (TFAM) (13-16). Activation of these downstream transcription factors is important for regulation of mitochondrial biogenesis (13). Exercise is a potent stimulator of PGC-1 α mRNA expression in skeletal muscle (17, 18). PGC-1 α transcription is

modulated by the binding of four different transcription factors to the PGC-1 α promoter region, cAMP response element-binding protein (CREB), activating transcription factor 2 (ATF2), MEF2, and forkhead box class-O (FoxO1) (19). Binding of these transcription factors is controlled by several different stimuli, many of which are related to acute exercise. Exercise-induced nerve stimulated calcium signaling activates both Ca²⁺/calmodulin-dependent protein kinase IV (CaMKIV) and Calcineurin A. CaMKIV directly phosphorylates and activates CREB, while Calcineurin A phosphorylates MEF2, leading to increased binding of both to the promoter region of the PGC-1 α gene (16, 20). Exercise also activates p38 mitogen-activated protein kinase (p38 MAPK), which phosphorylates both MEF2 and ATF2 (21). Insulin signaling also impacts expression of PGC-1 α , although this relationship is not entirely understood. Insulin has been reported to increase PGC-1 α transcription (22), however, insulin also activates the Akt pathway, which in turn phosphorylates FoxO1 and inhibits transcription of PGC-1 α (23).

It had been long assumed that increases in mitochondrial biogenesis observed following exercise were attributed to an increase in PGC-1 α mRNA (18). However, more recent evidence suggests that exercise-induced mitochondrial biogenesis begins before the exercise-induced increase in PGC-1 α mRNA expression (24), and that activation of existing PGC-1 α protein is responsible for exercise-induced mitochondrial biogenesis, while the observed increase in mRNA expression is a compensatory mechanism to prepare for adaptations to future exercise bouts (25, 26). In order to exert its co-activating effect on mitochondrial biogenesis-related transcription, PGC-1 α must be translocated to the nucleus. At rest, the majority of PGC-1 α protein is found in the cytosol, however, following endurance exercise, nuclear PGC-1 α content increases dramatically without a change in total intra-cellular PGC-1 α , suggesting a translocation from the cytosol to the nucleus (25). Once inside the nucleus, the de-acetylase sirtuin-1 (SIRT1) acts to de-acetylate PGC-1 α , activating it, and allowing it to bind to downstream transcription factors. This PGC-1 α -transcription factor complex then binds to DNA and initiates transcription of genes related to mitochondrial biogenesis (26-28).

Expression of PGC-1 α mRNA following exercise has been shown to be temperature sensitive in humans (11). PGC-1 α mRNA response to exercise is blunted following exercise in a hot environment as compared to room temperature. However, several other genes associated with mitochondrial biogenesis are not affected by temperature (11). Currently, the post-translational mechanisms of PGC-1 α protein after exercise in different environmental temperatures is not known. Understanding the stimulus that temperature and exercise have on the regulation of mitochondrial biogenesis is important in order to optimize training programs to be more effective at increasing performance, maintaining optimal function, and treating individuals with mitochondrial dysfunction. Therefore, the purpose of this study is to determine the acute response of PGC-1 α , both transcriptionally and post-translationally, to exercise in a hot (33 °C), compared to a room temperature (20 °C) environment. More specifically, this study aims to determine how the normally observed translocation of PGC-1 α protein into the nucleus, as well as binding of CREB, ATF2, MEF2, and FOXO1 to the PGC-1 α -promoter region of chromosomal DNA, is altered following exercise in a hot environment compared to a room temperature control environment.

Methods

Participant Screening. Recreationally-active males ($n = 11$) between the ages of 19 and 45 were recruited as participants in this study. Participants were informed of the general methods and procedures, risks and benefits associated with participating in the study, the measures taken to minimize risk, and a complete description of their rights as a volunteer. Participants signed an Institutional Review Board (IRB) approved Informed Consent form. All potential participants were stratified for risk according to ACSM guidelines. Only participants stratified as “low risk” for participation in physical activity were enrolled in the study.

Aerobic Capacity Tests. Participants performed a graded exercise test to measure aerobic capacity and to establish cycling intensity for the trial sessions. Participants cycled on a magnetically braked Velotron cycle ergometer (Racermate, Seattle, WA) beginning at a workload of 95 watts (W), and the workload was increased by 35 W every 3 min until volitional fatigue. Expired gases were analyzed using a flow and gas concentration calibrated TrueOne 2400 metabolic cart (ParvoMedics, Sandy, UT). $\text{VO}_2 \text{ peak}$ was defined as the highest 15 s average VO_2 during the protocol. W_{max} was calculated by taking the time completed in the last stage divided by the total stage duration (3 min) multiplied by 35 W, and added to the W of the last completed stage.

Body Composition Assessments. For descriptive purposes, participants had their body composition determined by hydrostatic weighing using an electronic load cell based system (Exertech, Dresbach, MN) corrected for estimated residual lung volume (29). Body density from hydrostatic weighing was converted to percent body fat using the Siri equation (30).

Experimental Trials. Each participant completed two trials, using a randomized, counterbalanced cross-over design. Trials consisted of 60 min of cycling at 60% of W_{max} in either a room temperature (C) environment (20°C , 60 % humidity), or hot (H) environment (33°C , 60 % humidity). This exercise protocol was developed to control for total workload, while also ensuring that recreationally-trained participants

would be able to complete the exercise protocol in the hot condition. Following exercise, participants recovered in a supine position for 3 h at room temperature. Both trials were performed in a temperature and humidity controlled environmental chamber (Darwin, St Louis, MO), separated by at least 5, and no more than 20, days. On the trial day, participants were instructed to arrive at the laboratory after a 8 h fast, having replicated an identical 24 h diet which included abstaining from caffeine, alcohol, and tobacco prior to both trials. Additionally, participants were instructed to log any physical activity in the 48 h prior to each trial, and asked to abstain from exercise in the 24 h preceding each trial. Participants ingested a Jonah Core Body Temperature Capsule with 125 mL of water and a fiber bar (29g carbohydrate, 9g fiber, 4g fat, 2g protein. Fiber One, General Mills, Minneapolis, MN) to aid in the capsule passing through the stomach and into the small intestine. 1 h after ingestion of the core temperature capsule, participants began the exercise protocol in the environmental chamber at the appropriate temperature. The 3 h recovery period began immediately upon completion of the cycling bout.

Muscle Biopsies. Muscle biopsies were taken pre-, post-, and 3 h post-exercise in each trial. Biopsies were taken from the *vastus lateralis* muscle using a 5 mm Bergstrom percutaneous muscle biopsy needle with the aid of suction. Each subsequent biopsy during a trial was obtained from the same leg using a separate incision 2 cm proximal to the previous biopsy. After excess blood, connective tissue, and fat were quickly removed, tissue samples were divided into two parts. One was immersed in RNAlater (Qiagen, Valencia CA) for mRNA analysis, and stored at 4° C overnight, then at -80° C until further analysis. The remaining tissue was flash frozen in liquid nitrogen, and stored at -80° C for later analysis.

Body core temperature, skin temperature, and heart rate. Body core temperature was measured by a Jonah Core Body Temperature Capsule ingestible thermistor and transmitted telemetrically to an EQ02 LifeMonitor Sensor Electronics Module (SEM; Hidalgo Limited, Cambridge, UK) every 15 s. The EQ02 LifeMonitor SEM was also used to measure skin temperature (using an infrared thermistor) and heart rate,

both of which were also logged every 15 s. Data were averaged for the entire 60 min to represent the cycling bout.

Rating of perceived exertion. Rating of perceived exertion was assessed at min 15, 30, 45, and 60 of the cycling protocol using the 6-20 Borg Scale (31). An average of all 4 assessments was used to represent the entire cycling bout.

Gas collection. Expired gases were collected during exercise for determination of exercise intensity using a flow and gas concentration calibrated TrueOne 2400 metabolic cart (ParvoMedics, Sandy, UT). Gas was collected for 5 min at 10, 25, 40, and 55 min of the cycling protocol. The last 3 min of each segment were averaged to represent the collection period, and all 4 segments were averaged to represent the entire cycling bout.

mRNA Extraction. A 26.0 ± 10.8 mg piece of skeletal muscle was homogenized in 800 μl of Trizol (Invitrogen, Carlsbad CA) using an electric tissue disruptor (Tissue Tearor, Biosped Products Inc, Bartlesville OK). Samples were then incubated at room temperature for 5 minutes, after which 160 μl of chloroform was added. Tubes were shaken vigorously by hand for 15 seconds. After an additional incubation at room temperature for 2 to 3 minutes, the samples were centrifuged at 12,000 g for 15 minutes and the aqueous phase was transferred to a fresh 1.5 mL tube. mRNA was precipitated by adding 400 μl of isopropyl alcohol and incubated overnight at -20°C . The RNA was purified using an RNeasy mini kit (Qiagen, Valencia CA, Cat#74104) according to the manufacturer's protocol using the additional DNase digestion step (RNase-free DNase set, Qiagen, Valencia CA). RNA was then quantified using a nanospectrophotometer (nano-drop ND-1000, Wilmington DE). RNA integrity was assessed using an Agilent RNA 6000 Kit (Agilent Technologies, Santa Clara, CA) according to manufacturer instructions, and read on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). These quality control measures confirmed pure, intact RNA (260:280 ratio = 2.02 ± 0.13 ; 260:230 ratio = 1.56 ± 0.33 ; RIN = 8.0 ± 0.8).

cDNA Synthesis. First-strand cDNA synthesis was achieved using Superscript-first-strand synthesis system for RT-PCR kit (Invitrogen, Carlsbad CA) according to the manufacturer's protocol. The resulting cDNA will then diluted with the appropriate amount of RNase free water to achieve a final cDNA concentration of 0.5 µg/µL in the PCR reaction.

qRT-PCR. Each 20 µl qRT-PCR reaction contained 500 nM primers, 250 nM probe (PimeTime qPCR assay, Integrated DNA Technologies, Coralville, IA), 10 µL of Brilliant III Ultra-Fast QPCR Master Mix (Agilent Technologies, Santa Clara, CA), and 2.5µl of sample cDNA. PCR was run using a Stratagene mx3005p PCR system (Aligent Technologies, Santa Clara, CA) using a 2-step Roche protocol (1 cycle at 95 °C for 3 minutes, followed by 40 cycles of 95 °C for 5 seconds followed by 60 °C for 20 seconds). Quantification of mRNA for genes of interest was calculated on post- and 3 hour post-exercise muscle samples relative to pre-exercise and stable reference genes using the $2^{-\Delta\Delta CT}$ method (32, 33). The geometric mean of the most stable reference genes for each participant, as determined by qbase+ geNorm software (Biogazelle, Zwijnaarde, Belgium) was used to calculate fold-changes of the genes of interest according to the $2^{-\Delta\Delta CT}$ method (34). Fold-change values were log transformed for statistical analysis to yield a normal distribution, as fold-change data is heavily skewed in a linear scale (34).

Sub-cellular fractionation. Sub-cellular fractionation was performed using an NE-PER Nuclear and Cytosolic Extraction Kit (Pierce Biotechnologies, Rockford, IL) according to manufacturer instructions, with additional wash steps to purify the nuclear extract. Briefly, 22.9 ± 1.3 mg of muscle was washed with 500 µL of phosphate buffered saline (PBS), and centrifuged at 500x g for 5 minutes at 4° C. Samples were then homogenized in 200 µL of cytoplasmic extraction reagent I supplemented with HALT protease inhibitors (Pierce Biotechnologies, Rockford, IL) using an electronic tissue disruptor (Tissue Tearor, Biosped Products Inc, Bartlesville OK), vortexed for 15 seconds, and incubated on ice for 10 minutes. Next, 11 µL of ice cold cytoplasmic extraction reagent II was added, tubes were vortexed, and spun in a centrifuge

at 16,000x g for 5 minutes at 4° C to elute the cytoplasmic fraction. The supernatant (the cytosolic fraction) was immediately transferred to a pre-chilled 1.5 mL tube and stored at -80° C for later analysis. The pellet containing the nuclear fraction was washed 4 times with PBS to eliminate any cross-contamination from the cytosolic extract. The pellet was then suspended in 100 µL of nuclear extraction reagent supplemented with HALT protease inhibitors, and incubated on ice for 40 minutes, vortexing every 10 minutes. The nuclear extract was eluted by centrifuging at 16,000x g for 10 minutes at 4° C, transferred to a pre-chilled 1.5 mL tube and stored at -80° C for later analysis. This protocol resulted in less than 2% cross-contamination between fractions, as evidenced by a high abundance of lactate dehydrogenase (LDH) and lack of nuclear matrix protein 84 (NMP84) in cytosolic fractions, and an absence of LDH and presence of NMP84 in nuclear fractions.

Protein quantification. Protein quantity of each fraction was measured using a commercially available BCA Protein Assay Kit (Pierce Biotechnologies, Rockford, IL) according to manufacturer instructions. Absorbance of samples was read at 562 nm using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA). Protein concentrations were then calculated using a standard curve according to kit instructions. Sample concentrations were $2802.1 \pm 380.3 \text{ } \mu\text{g} \cdot \mu\text{L}^{-1}$.

Gel electrophoresis and western blotting. Proteins were separated by SDS-PAGE. 20 µg of total protein was added to each well of pre-cast 4-20% polyacrylamide gels (NuSep, Homebush, New South Wales, Australia), along with 7.5 µL of 4x loading buffer (LiCor, Lincoln, NE) containing β-mercaptoethanol (1 µL per µL of 4x loading buffer), and an appropriate volume of sample buffer so that each well contained 30 µL. Gels were run at 150 V for 1 h in a Mini Trans-Blot tank (Bio-Rad, Hercules, CA). Gels were then be transferred to a nitrocellulose membrane using a Trans-Blot Turbo transfer system (Bio-Rad, Hercules, CA). The membrane was then be blocked for 1 h at room temperature with LiCor blocking buffer (LiCor, Lincoln, NE), and incubated overnight at 4° C in rabbit anti-human primary antibody for either PGC-1α, LDH, or NMP84 (all primary antibodies from Santa Cruz Biotechnology, Santa Cruz, CA) in a tube rotator.

The next day, the membrane was washed with Tris-buffered saline plus Tween 20 (TBST) 3 times for 5 minutes each, then incubated in secondary antibody using 1 μ L LiCore goat anti-rabbit antibody in 5 mL of blocking buffer for 1 h. The membrane was again washed with TBST 3 times for 5 minutes each, and imaged by infrared fluorescence using an Odyssey Fc imaging system (LiCor, Lincoln, NE). Bands were quantified using Image Studio 5.2 software (LiCor, Lincoln, NE).

Chromatin immunoprecipitation. Binding of CREB, ATF2, MEF2, and FoxO1 to the PGC-1 α promoter region was assessed by chromatin immunoprecipitation (ChIP). There was sufficient sample from 9 participants for ChIP analysis ($n = 9$). ChIP was performed using a commercially available EpiQuick Tissue Chromatin Immunoprecipitation Kit (Epigentek, Farmingdale, NY) according to manufacturer instructions. Briefly, protein-DNA interactions were fixed by cross-linking 24.7 ± 4.9 mg of skeletal muscle with 1% formaldehyde. Samples were then homogenized with an electronic tissue disruptor, and DNA was sheared by sonication. Shearing was accomplished by sonicating samples 4 pulses of 15 to 20 seconds with a Q55 sonicator (Qsonica, Newtown, CT) set to an amplitude of 40, with at least 1 minute on ice between pulses. This protocol resulted in average DNA fragment sizes of 400 to 1,200 base pairs. Fragment sizes were checked using an Agilent DNA 7500 Kit (Agilent Technologies, Santa Clara, CA) according to manufacturer instructions, and read on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Protein-bound DNA was immunoprecipitated using Normal Mouse IgG (negative control), anti-RNA Polymerase II (positive control), or anti-CREB, ATF2, MEF2, or FOXO1 antibodies (all antibodies from Santa Cruz Biotechnology, Santa Cruz, CA). Cross-linking was reversed using Proteinase K, and DNA was purified using spin columns and 90% EtOH. Purified immunoprecipitated DNA was quantified using qRT-PCR as outlined above. Custom probe/primer pairs were designed for putative binding sites of each transcription factor within the PGC-1 α promoter region (PimeTime qPCR assay, Integrated DNA Technologies, Coralville, IA), as well as GAPDH to quantify input DNA. Raw PCR data was normalized using the percent input method (35), in which recovered immunoprecipitated DNA is expressed as a percentage of total DNA input into the reaction.

Statistical Analysis. Differences in exercise VO₂, HR, core temperature, skin temperature, and RPE were analyzed using paired t-tests. Differences in gene expression, protein content, and chromatin binding throughout the three time points (pre-, post- and 3 h post-exercise) and between the 2 trials (Control and Hot) were analyzed using 3x2 repeated measures ANOVAs (time x trial). In the event of a significant F-ratio, Fishers protected least significant difference method was used to detect where differences occurred. A probability of type I error of less than 5% was considered significant ($p < 0.05$). All data are reported as mean \pm SE.

Results

Participant and exercise descriptive data. Recreationally-trained males ($n = 11$, age: 24 ± 3 y, height: 178 ± 5 cm, weight: 80.3 ± 12.8 kg, percent body fat: $14.6 \pm 3.6\%$, $\text{VO}_2 \text{ peak}$: $4.34 \pm 0.84 \text{ L} \cdot \text{min}^{-1}$, W_{\max} : 275 ± 40 W) completed this study. VO_2 ($p = 0.003$), percent $\text{VO}_2 \text{ peak}$ ($p = 0.010$), heart rate ($p < 0.001$), skin temperature ($p < 0.001$), and RPE ($p < 0.001$) were all higher in H than C, while core body temperature was not different between trials ($p = 0.172$). Exercise descriptive data is presented in table 1.

Mitochondrial biogenesis related and transcription factor mRNA expression. PGC-1 α , ERR α , and VEGF mRNA increased following exercise in C ($p = 0.002$, $p = 0.011$, and $p < 0.001$, respectively). PGC-1 α and VEGF mRNA also increased following exercise in H, but to a lesser degree than in C ($p = 0.037$ and $p = 0.008$, respectively). ERR α did not increase after exercise in H and was lower following H than C ($p = 0.007$). NRF-1 was unchanged following exercise in C, but was down-regulated following exercise in H ($p = 0.002$). GABPA and SIRT-1 mRNA were lower following H than C ($p = 0.046$ and $p = 0.021$, respectively). PPARG and TFAM mRNA were unaffected by exercise in either H or C ($p = 0.393$ and $p = 0.702$, respectively). Mitochondrial biogenesis-related gene expression data is presented in Figure 1. CREB and ATF2 mRNA were unaffected by exercise in either H or C ($p = 0.473$ and $p = 0.333$, respectively). MEF2 was down-regulated following exercise in H ($p = 0.037$), but was unchanged in C ($p = 0.218$). FoxO1 was not different between trials ($p = 0.724$), but was increased with exercise ($p < 0.001$). PGC-1 α promoting transcription factor gene expression data is presented in Figure 2.

CREB, ATF2, MEF2, and FoxO1 DNA binding. Binding of CREB to the PGC-1 α promoter increased post- ($p = 0.026$) and 3 h post-exercise ($p = 0.011$) in C, but did not increase in H. CREB binding was significantly lower post- ($p = 0.005$) and 3 h post-exercise ($p = 0.004$) in H as compared to C (Figure 3). ATF2 binding to the PGC-1 α promoter was not different between trials ($p = 0.124$), but was increased post- ($p = 0.017$) and 3 h post-exercise (0.014 ; Figure 3). MEF2 binding to the PGC-1 α promoter increased post-exercise in C ($p = 0.045$), but was not significantly different from pre-exercise at 3 h post-exercise in C ($p = 0.105$).

Conversely, MEF2 binding did not increase from pre- to post-exercise in H ($p = 0.688$), but was higher 3 h post-exercise than both pre- and post-exercise ($p = 0.045$ and $p = 0.03$, respectively; Figure 3). FoxO1 binding to the PGC-1 α promoter was increased post- ($p = 0.035$) and 3 h post-exercise ($p = 0.036$) in C, but did not increase in H. FoxO1 binding was significantly higher in C than H post-exercise ($p = 0.006$; Figure 3).

Sub-cellular location of PGC-1 α protein. At rest, $77.5 \pm 5.2\%$ of PGC-1 α was present in the cytosol (Figure 4A). Post-exercise, there was a ~20% increase in nuclear PGC-1 α ($p = 0.029$; Figure 4B), without a change in total PGC-1 α ($p = 0.258$), indicating a translocation of cytosolic PGC-1 α into the nucleus. There were no differences between trials in total ($p = 0.523$), cytosolic ($p = 0.333$), or nuclear ($p = 0.602$) PGC-1 α (Figure 4B-D). Efficiency of sub-cellular fractionation was demonstrated by a high abundance of lactate dehydrogenase (LDH) and lack of nuclear matrix protein 84 (NMP84) in cytosolic fractions, and an absence of LDH and presence of NMP84 in nuclear fractions (Figure 4E). Nuclear protein yield was not impacted by exercise, as demonstrated by equal amounts of NMP84 in pre-, post- and 3h post-exercise samples (Figure 4F).

Discussion

The main findings of this study were that PGC-1 α mRNA expression, as well as expression of several other mitochondrial biogenesis-related genes, is blunted following exercise in a hot environment, coinciding with decreased binding of CREB, MEF2, and FoxO1 to the PGC-1 α promoter region. However, translocation of PGC-1 α protein from the cytosol into the nucleus following exercise was unaffected by the temperature intervention. These data demonstrate somewhat of a paradox, as the presence of PGC-1 α in the nucleus should allow it to exert its co-transcriptional activity. Yet, several genes co-activated by PGC-1 α , including ERR α , MEF2, and NRF-1, were down-regulated following exercise in the heat. Overall, these data indicate exercise in the heat has a deleterious effect transcription factor binding at the PGC-1 α promoter, which could potentially inhibit mitochondrial biogenesis.

PGC-1 α mRNA expression following acute exercise has previously been shown to be intensity-dependent (17, 36). Based on these findings alone, it may be expected that PGC-1 α mRNA would increase to a greater degree following exercise in the heat, as at a given workload, relative exercise intensity is likely higher in the heat. Unsurprisingly, there was a higher relative exercise intensity at the clamped workload in the heat in the present study, as demonstrated by higher VO₂, HR and reported RPE. However, the differences in relative exercise intensity in the present study were relatively small, and certainly did not augment PGC-1 α expression in the heat. Rather, these data support earlier findings of blunted PGC-1 α mRNA expression following exercise in the heat (11).

The potential mechanism through which PGC-1 α mRNA expression is blunted following exercise in the heat was previously unknown. To explore potential avenues through which this may occur, chromatin immunoprecipitation was carried out to assess binding of the transcription factors CREB, ATF2, MEF2, and FoxO1 to the promoter region of the PGC-1 α gene. Binding of CREB, MEF2, and FoxO1 were all altered following exercise in the heat. CREB binding was lower both immediately post and 3 hours post exercise in the hot trial. CREB has been shown to be activated in response to cold temperatures through the

β 3-adrenergic receptor (37, 38), however, it is currently unknown whether this pathway would be further down-regulated in response to hot temperatures. If so, it may explain the lower amount of CREB binding observed in the hot trial, as skin temperature was significantly higher in the hot trial than the control trial. MEF2 and FoxO1 binding were increased post exercise in the control trial, and not in the hot trial. However, neither MEF2 nor FoxO1 binding was different between the two trials 3 hours post-exercise. Heat stress has been shown to upregulate Akt activity in rats (39) and swine (40). Akt-mediated phosphorylation excludes FoxO1 from the nucleus, therefore inhibiting its DNA binding (23). This mechanism could explain decreased FoxO1 binding post-exercise in the hot trial. By 3 hours post-exercise, FoxO1 binding was not different between the trials, as participants had been recovering at room temperature. This finding could indicate an altered time course of normally observed PGC-1 α expression following exercise in the heat. It is possible that rather than a blunting of mitochondrial biogenesis-related gene expression, there is a delay in their onset, and further time-course investigation is warranted. Additionally, it is unknown whether the decreased transcription factor binding observed in the present study is a general phenomenon, or specific to this pathway.

At rest, the majority of PGC-1 α resided in the cytosol, and following exercise, there was an increase in nuclear PGC-1 α without an increase in total PGC-1 α , indicating a translocation of PGC-1 α into the nucleus. While there was not a corresponding decrease in cytosolic PGC-1 α , this could be explained by the fact that with approximately 4 times as much PGC-1 α in the cytosol, a comparatively small decrease in concentration is less likely to be detected than the ~20% increase in the nuclear fraction. The observed sub-cellular location of PGC-1 α at rest and following exercise is in agreement with previous work (25, 28). While the present study displays a smaller increase in nuclear PGC-1 α post-exercise than previously reported (~20% in the present study vs. ~32% reported by Gurd, et al. and ~54% reported by Little, et al.), this may be due to slight differences in the exercise protocol or training status of the participants.

While the translocation of PGC-1 α to the nucleus was not impaired by exercise in the heat, the transcription of several of its downstream targets, including ERR α , MEF2, and NRF-1 were down-regulated following exercise in the heat. Additionally, while GABPA expression was not down-regulated, it was blunted following exercise in the heat. PGC-1 α co-activates the ERR α and GABPA, which each contain binding sites in their own promoter regions (41, 42), which results in an autoregulatory loop. Additionally, while MEF2 modulates transcription of PGC-1 α , PGC-1 α also increases transcription of MEF2, creating an additional autoregulatory loop (43). With PGC-1 α present in the nucleus, it would be expected that transcription of each of these targets would increase, which was observed in the control condition. However, following exercise in the heat, this relationship was disrupted. One potential mechanism through which this paradox may be explained is the de-acetylase SIRT1. SIRT1 de-acetylates PGC-1 α , activating it (26). SIRT1 activity is increased with exercise and linked to increased mitochondrial biogenesis (27, 28). Recently, SIRT1 has been shown to decline after heat shock in HCT116 cells (44). While it is unknown how SIRT1 activity responds to heat stress in a human model, a potential for a decrease in SIRT1 following exercise in the heat would explain the blunted transcription of transcription factors downstream of PGC-1 α , even when present in the nucleus.

Directions for future research include determination of whether the blunting of mitochondrial biogenesis-related gene expression following exercise in the heat is truly a deleterious effect, or if a time-delayed, but intact, response of these genes is observed over a recovery period of greater than 3 hours. It remains possible that through an unknown mechanism, the time-course of mitochondrial biogenesis-related gene expression is impacted by exercise in the heat, rather than blunted. One potential mechanism for the observed blunting of mitochondrial biogenesis-related genes following exercise in the heat is a decrease in SIRT1 activity, although the response of SIRT1 to exercise in the heat has not been categorized. Additionally, the chronic impact of exercise in the heat on mitochondrial biogenesis needs to be explored. It is unknown whether the acute response observed in the present study will lead to reduced mitochondrial biogenesis over a period of exercise training in the heat, or whether there will be some type of adaptation, or ‘muscular heat

acclimation' that allows for the acute response of mitochondrial biogenesis-related gene expression to return to a more normal profile following repeated bouts of exercise in a hot environment. This possibility is especially intriguing given the finding that translocation PGC-1 α protein into the nucleus, where it can exert its co-activating activity, was unaffected by exercise in the heat as compared to room temperature, despite the observed blunting of mitochondrial biogenesis-related gene expression. Based on this paradox, a study of longer-term muscular adaptation to exercise in the heat is warranted.

In conclusion, through decreased binding of CREB, MEF2, and FoxO1 to the promoter region of the PGC-1 α , gene, expression of PGC-1 α , as well as expression of several other mitochondrial biogenesis-related genes, is blunted following exercise in the heat as compared to a room temperature control. This blunted effect occurred despite the translocation of PGC-1 α into the nucleus following exercise being no different between the hot and control conditions, thus indicating that a different mechanism, potentially de-acetylation of PGC-1 α by SIRT-1, is involved in the observed blunting of mitochondrial biogenesis-related gene expression. Together, these data demonstrate that exercise in the heat may be deleterious for mitochondrial biogenesis.

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Tables and Figures

Table 1. Exercise descriptive data. [†] p < 0.05 from control.

Trial	VO ₂ (L · min ⁻¹)	% VO ₂ peak	HR (bpm)	Skin temp (° C)	Core temp (° C)	RPE
Control	2.84 ± 0.38	64.5 ± 6.0	156 ± 10	32.3 ± 1.1	37.8 ± 0.3	12 ± 1
Hot	3.05 ± 0.46 [†]	69.7 ± 5.1 [†]	166 ± 9 [†]	36.6 ± 0.7 [†]	38.0 ± 0.3	15 ± 1 [†]

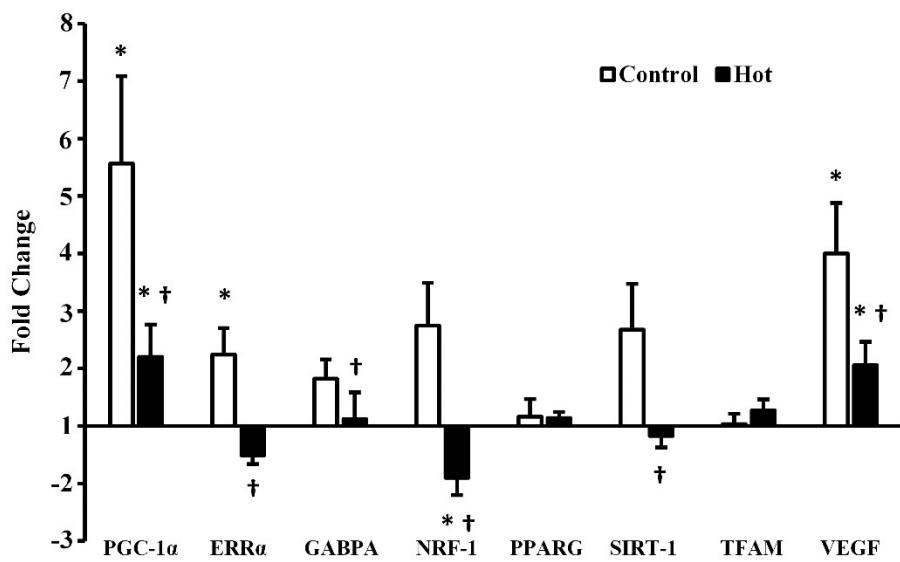


Figure 1. mRNA expression of mitochondrial biogenesis related genes 3 h post-exercise. * p < 0.05 from pre-exercise, † p < 0.05 from control.

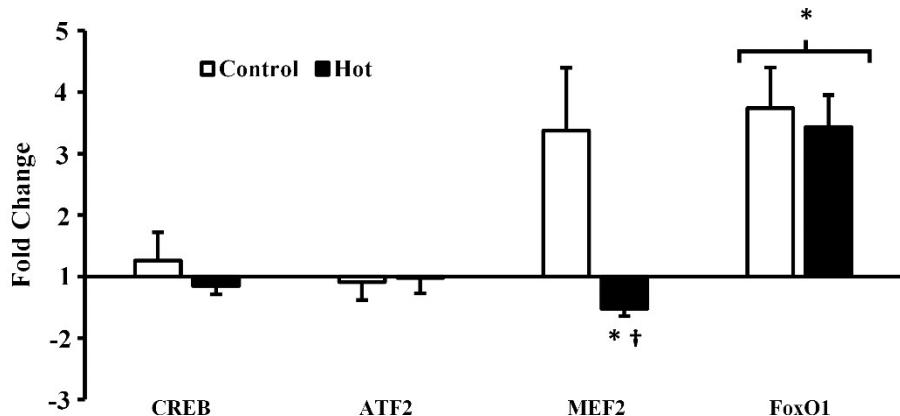


Figure 2. mRNA expression of transcription factors 3 h post-exercise. * $p < 0.05$ from pre-exercise, † $p < 0.05$ from control.

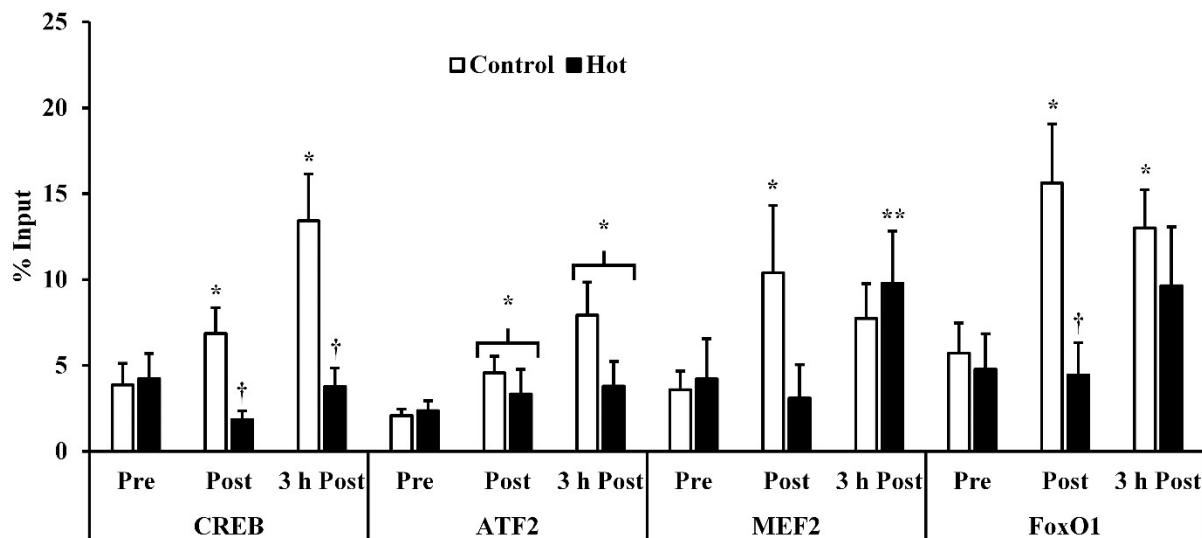
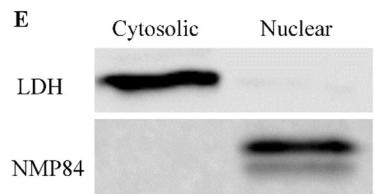
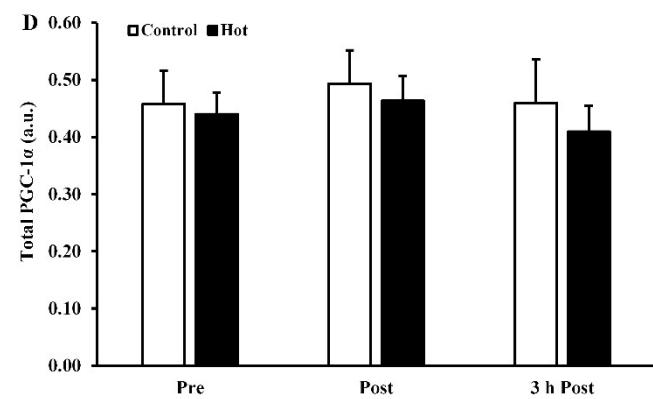
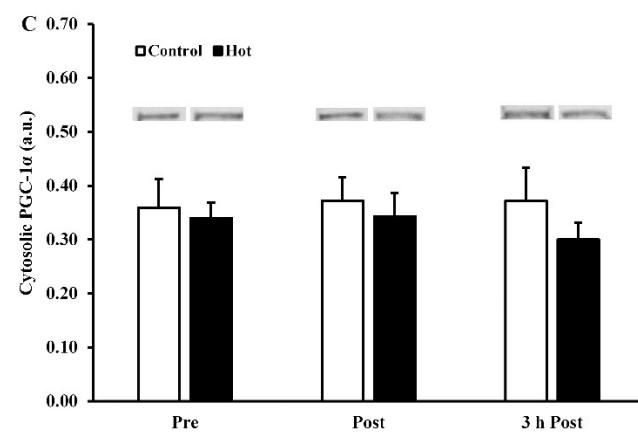
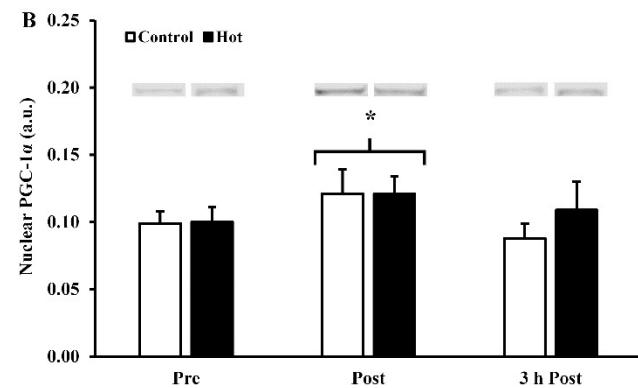
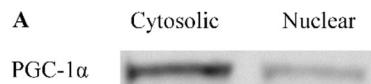


Figure 3. DNA binding of transcription factors to the PGC-1 α promoter. * p < 0.05 from pre-exercise, ** p < 0.05 from pre-exercise and post-exercise, † p < 0.05 from control.



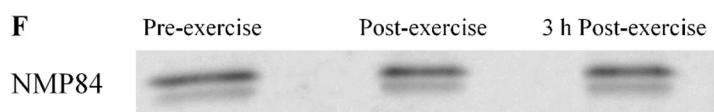


Figure 4. **A.** Sub-cellular location of PGC-1 α at rest. **B.** Nuclear PGC-1 α . **C.** Cytosolic PGC-1 α . **D.** Total PGC-1 α . **E.** Purity of cytosolic and nuclear fractions demonstrated by presence of LDH and NMP84. **F.** Nuclear protein yield, as demonstrated by NMP84. * p < 0.05 from pre-exercise.

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