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Acute simulated soccer-specific training increases PGC-1 α mRNA expression in human skeletal muscle

Tae-Seok Jeong¹, Jonathan D Bartlett¹, Chang-Hwa Joo¹, Jari Louhelainen², Graeme L Close¹, James P Morton¹, Barry Drust¹

¹Research Institute for Sport and Exercise Sciences
Liverpool John Moores University
Tom Reilly Building
Byrom St Campus
Liverpool
L3 3AF
UK

²Faculty of Pharmacy and Biomolecular Sciences
Liverpool John Moores University
Tom Reilly Building
Byrom St Campus
Liverpool
L3 3AF
UK

Running title: Soccer training and PGC-1 α mRNA expression

Address for correspondence:

Professor Barry Drust
Research Institute for Sport and Exercise Sciences
Liverpool John Moores University
No 70 Great Crosshall Street
Liverpool
L3 3AB
United Kingdom
Email: B.Drust@ljmu.ac.uk
Tel: 0151 231 4032

Abstract

The aim of the current study is to quantify oxygen uptake, heart rate and molecular responses of human skeletal muscle associated with mitochondrial biogenesis following an acute bout of simulated soccer training.

Muscle biopsies (vastus lateralis) were obtained from nine active men immediately pre-, post- and 3 h post-completion of a laboratory-based soccer-specific training simulation (LSSTS) on a motorised treadmill.

The LSSTS was a similar intensity ($55 \pm 6\% \dot{V}O_{2\max}$) and duration (60-min) as that observed in professional soccer training (e.g. standing 41%, walking 37%, jogging 11%, high-speed running 9% and sprinting 2%). Post-exercise, muscle glycogen decreased (Pre; $397 \pm 86 \text{ mmol}\cdot\text{kg}^{-1} \text{ dw}$, Post; $344 \pm 64 \text{ mmol}\cdot\text{kg}^{-1} \text{ dw}$; $P = 0.03$), plasma lactate increased ($P < 0.001$) up to $\sim 4\text{-}5 \text{ mmol}\cdot\text{L}^{-1}$, NEFA and glycerol increased ($P < 0.001$) to values of $0.6 \pm 0.2 \text{ mmol}\cdot\text{L}^{-1}$ and $145 \pm 54 \mu\text{mol}\cdot\text{L}^{-1}$, respectively. PGC-1 α mRNA increased ($P = 0.009$) 5-fold 3 h post-exercise.

We provide novel data by demonstrating that soccer-specific training is associated with increases in PGC-1 α mRNA. These data may have implications for practitioners in better understanding the specific 'muscle' responses to soccer-specific training protocols in the field.

Key Words: intermittent exercise, vastus lateralis, cell signalling, mitochondrial biogenesis

Introduction

The aerobic fitness of soccer players is a fundamental component of an individual's ability to repeatedly perform high-intensity activities during match-play (Stone & Kilding, 2009). One major aim of training in soccer should, therefore, be to enhance the aerobic fitness of players. Increases in aerobic performance following soccer-specific training are associated with both cardiovascular adaptations (Knoepfli-Lenzin et al., 2010) and skeletal muscle changes (Bangsbo, 1994; Krstrup, Christensen, et al., 2010b). Adaptations in skeletal muscle following exercise begin at the molecular level resulting in morphological and metabolic changes in mitochondria that increase the rate of energy production from both aerobic and oxygen-independent pathways (Hawley, Tipton, & Millard-Stafford, 2006). Such alterations in mitochondria include long-term changes in mitochondrial protein content and enzyme activities in skeletal muscle. For example, **soccer training in untrained populations are shown to** increase 3-hydroxyacyl co-enzyme A dehydrogenase (HAD), citrate synthase (CS) activity content and mean muscle fibre area and decreases the fraction of Type IIx fibres ((Bangsbo et al., 2010; Krstrup, Christensen, et al., 2010b). These adaptations are deemed important in underpinning the improved exercise capacity and greater resistance to fatigue observed following training.

Given the importance of the oxidative capacity to improve soccer specific performance, it is important to understand the process that drives this adaptive response. At a molecular level, the regulation of *training adaptation* appears to be controlled by the cumulative effects of transient increases in mRNA transcripts encoding mitochondrial proteins that follow each acute training session (Egan, O'Connor, Zierath, & O'Gorman, 2013; Perry et al., 2010). Upon the onset of contraction, alterations in various mechanical and metabolic characteristics initiate a host of primary signals (i.e. increased AMP/ATP ratio, Ca²⁺, reactive oxygen species (ROS), lactate, reduced muscle glycogen availability) that result in the activation of key signalling kinases that converge on the regulation of key transcription factors or co-activators. For example, the adenosine monophosphate-activated protein kinase (AMPK) and p38 mitogen activated protein kinase (p38MAPK) are suggested to be two important kinases that increase the activity of peroxisome proliferator-activated γ receptor coactivator (PGC-1 α). **Although a host of factors are deemed to play a role in the adaptation to training (Coffey & Hawley, 2007), PGC-1 α is regarded as the 'master regulator' controlling various processes of *training adaptation*, such as mitochondrial biogenesis, angiogenesis, antioxidant defence and inflammatory response (Egan et al., 2010; Jäger, Handschin, St-Pierre, & Spiegelman, 2007; Olesen et al., 2010; Wright, Geiger, Han, Jones, & Holloszy, 2007). Despite training adaptations occurring (albeit reduced) in the absence of functional PGC-1 α , i.e. PGC-1 α knockout models (REF), the importance of PGC-1 α is characterised in rodent models demonstrating that overexpression increases oxidative enzyme activity (Lin et al., 2002), insulin sensitivity (Benton, Wright, & Bonen, 2008) and improved exercise capacity (Calvo et al., 2008).**

It has been suggested that the molecular adaptations to training are highly specific to the type of training stimulus that is provided with factors such as volume, intensity, duration and mode of exercise all significant (Coffey & Hawley, 2007). These parameters are probably important as a consequence of the prominent disruptions to cellular metabolism in driving transcription. Recent years has seen a

move towards sports-specific conditioning rather than traditional athletic type training especially in sports such as soccer where the competitive demands restrict training time (Impellizzeri et al., 2006). This sports-specific conditioning frequently takes the form of structured technical and tactical practices that enable athletes to “train as you play” (Bishop, 2009). The physiological responses to these types of activities have been characterized to some extent (Jeong, Reilly, Morton, Bae, & Drust, 2011) though it is currently unclear what impact such soccer-specific training has on whole body metabolic responses and the complex signalling networks that may underpin the molecular changes in skeletal muscle associated with exposure to this type of exercise. Indeed, whereas we (Bartlett et al., 2012) and others (Gibala et al., 2009) have shown that both supra-maximal and near-maximal models of high-intensity interval exercise are capable of activating the aforementioned cell signaling pathways, it is currently unknown if the typical intensities, duration and activity profile of soccer-specific training is also sufficient to promote acute activation of those pathways that regulate an oxidative training adaptation.

The aim of the present study was therefore to investigate whole body metabolic responses and the acute signalling responses associated with *mitochondrial biogenesis* in skeletal muscle of healthy men in response to a single bout of soccer-specific simulated intermittent exercise. To this end, we devised a laboratory based soccer-specific training simulation (completed on a motorised treadmill) that included the main movement categories inherent to soccer-specific exercise. We hypothesised that performing soccer-specific intermittent exercise would induce increases in the phosphorylation of important protein kinases, thereby leading to activation of PGC-1 α mRNA expression.

Methods

Nine males who regularly participated in team sport volunteered in the study (mean \pm SD: age, 25 ± 4 yr; body mass, 75 ± 7 kg; height, 1.75 ± 0.04 m; $\dot{V}O_{2\max}$ 59 ± 6 ml \cdot kg $^{-1}\cdot$ min $^{-1}$). The experimental procedures and potential risks related to the study were explained verbally to all subjects. Written participant information was also given during a familiarization session. Informed consent was obtained from all subjects prior to participation. Subjects refrained from strenuous exercise at least 48 h before the exercise trial. None of the subjects had any current medical problems that were neurological and/or musculoskeletal or were under pharmacological treatment during the course of the study. The study was approved by the local Ethics Committee of Liverpool John Moores University.

Assessment of physiological fitness

All participants completed a $\dot{V}O_{2\max}$ test using an incremental exercise test performed on a motorised treadmill (HP cosmos Pulsar[®] 4.0, h/p/cosmos, Germany). Oxygen uptake was measured continuously during exercise using an on-line gas analysis system (Cortex Metamax, Leipzig, Germany). The test began with a 3-min warm up stage at a treadmill speed of 10 km \cdot h $^{-1}$ followed by 3 min stages at 12 km \cdot h $^{-1}$, 14 km \cdot h $^{-1}$ and 16 km \cdot h $^{-1}$. Upon completion of the 16 km \cdot h $^{-1}$ stage, the treadmill was inclined by 2 % every 3 min until volitional exhaustion. The $\dot{V}O_{2\max}$ was taken as the highest $\dot{V}O_2$ value obtained in any 10-s period and was stated as being achieved by the following end-point criteria: 1) heart rate within 10 b \cdot min $^{-1}$ of age-predicted maximum, 2) respiratory exchange ratio > 1.1 , and 3) plateau of oxygen consumption despite increased workload (Gilman, 1996).

Experimental Design

At least 3 days after the initial assessment of maximal oxygen consumption ($\dot{V}O_{2\max}$), subjects performed a single bout of a laboratory-based soccer-specific training simulation (LSSTS) on a motorised treadmill, which consisted of 3 identical 20-min blocks to create a 60 min protocol. Heart rate (HR) (Polar S610i, Kempele, Finland) and oxygen consumption (Cortex Metamax, Leipzig, Germany) were measured continuously during the exercise protocol and ratings of perceived exertion (RPE CR 1-10) (Borg, 1970) were measured every 20 min throughout exercise and 30 min after completion of the LSSTS. Blood samples were obtained pre-exercise, every 20-min during exercise, post-exercise and 3 h post-exercise. Muscle biopsies were taken from the vastus lateralis pre-, post- and 3 h post LSSTS. During the recovery period between the post-exercise biopsy and 3-h biopsy, subjects remained seated in the laboratory.

Exercise protocol

The development of the Laboratory Soccer Specific Training Simulation (LSSTS) has been previously described and validated (Jeong, Reilly, Morton, Drust 2013). In brief, the LSSTS attempted to recreate the physiological demands imposed on professional soccer players during an 'in-season' soccer

training session. The laboratory-based soccer-specific training simulation was devised for a motorized treadmill (HP cosmos Pulsar® 4.0, h/p/cosmos, Germany) for researchers interested in examining the specific metabolic and molecular responses to football training. Whilst it may be 'more realistic' to study a 'real pitch based' training session, obtaining muscle biopsies in the field is logistically problematic owing to issues of accurate sampling time points for multiple participants as well as ensuring safe and ethically approved locations for minor procedures.

The proportion of time designated for each discrete activity-category was the same as those recorded in the actual training session and the movements included consisted of walking, jogging, high speed running and sprinting. Static pauses were also included in which subjects remained stationary on the treadmill. A small portion of the activities in the 'field' training session involved utility activities such as sideways (2.1%) and backward movements (2%), however, these types of movements were not incorporated within the protocol due to the technical limitations of the equipment used and the safety issues associated with including these activities. As a consequence of these omissions, the sum of the percentage time in these two movements in training was added to the walking and jogging categories included in the protocol. The speeds of each movement on the treadmill were based on previous observations obtained during match play (Mohr, Krstrup, & Bangsbo, 2003). The relevant speeds utilised for walking, jogging, running and high-speed running were $\sim 6 \text{ km}\cdot\text{h}^{-1}$, $\sim 12 \text{ km}\cdot\text{h}^{-1}$, $\sim 19 \text{ km}\cdot\text{h}^{-1}$ and $\sim 23 \text{ km}\cdot\text{h}^{-1}$ respectively. The duration of each discrete bout of activity in the protocol was closely matched to those observed during the training session (Table 1). This permitted the total number of bouts for each discrete activity in the simulation to be calculated. A block of exercise incorporated 93 discrete activities that included 26 static pauses, 28 walks, 17 jogs, 16 high-speed runs and 6 sprints. The LSSTS thus incorporated a total 279 activities within 3-repeated identical blocks. Once the total numbers of bouts in each activity category were established, the order of the presentation of activities was determined. High-intensity activities were separated by low-intensity recovery periods to replicate the cyclical nature of the movement patterns observed in the training session. The time required to complete all speed changes between the different activity categories was monitored following the development of the exercise protocol for the simulation. This enabled the total time for the speed transitions included in the treadmill protocol to be calculated. The total time for changing speeds between the different categories of activities was 336 s during each block. The final duration of a block was then determined by summing *the total exercise time* that would be performed at constant speeds of each activity and *the total transition time* that was required to change between speeds. As a result a block of exercise lasted 20 minutes 36 seconds (Figure 1). This block was then repeated a total of 3 times, thereby resulting in the total protocol time closely resembling the total duration of the training session (61 min 48 seconds).

Dietary control

Subjects were instructed to follow their habitual diet for the 48 h preceding the LSSTS. Subjects refrained from ingesting any alcohol and caffeine for at least 48 h prior to the testing session and

subjects were required to attend the laboratory on the morning of testing in a fasted state. In the 30 min preceding exercise, subjects consumed a volume of water ($5 \text{ ml}\cdot\text{kg}^{-1}$). During exercise subjects were not allowed to drink. Only plain water was provided during the recovery period between the post-exercise and 3 h biopsies. This was calculated to compensate the loss of body fluid during exercise ($1.5\text{L} \times \text{loss of body mass (kg)}$).

Muscle biopsies

Muscle biopsies were obtained from separate incision sites (2-3 cm apart) from the lateral portion of the vastus lateralis muscle pre-, post- and 3 h after the exercise protocol using a Bard Monopty Disposable Core Biopsy Instrument 12 gauge x 10 cm length (Bard Biopsy Systems, Tempe, AZ, USA). Samples were obtained (four passes per biopsy each containing approximately 20-30 mg per pass) under local anaesthesia without epinephrine (0.5 % Marcaine, Astrazeneca, USA). They were immediately frozen in liquid nitrogen and stored at $-80 \text{ }^{\circ}\text{C}$ for later analysis.

Muscle analysis

Muscle Glycogen

Approximately 2-3 mg (*i.e. one pass*) of freeze-dried sample was powdered and subsequently hydrolyzed by incubation in 500 μl of 1 M HCl for 3-4 hr at 100°C . After cooling to room temperature, samples were neutralized by the addition of 250 μl $0.12 \text{ mol}\cdot\text{L}^{-1}$ Tris- $2.1 \text{ mol}\cdot\text{L}^{-1}$ KOH saturated with KCl. Following centrifugation, 150 μl of supernatant was analysed in duplicate for glucose concentration according to the hexokinase method using a commercially available kit (GLU-HK, Randox Laboratories, Antrim, UK). Glycogen concentration is expressed as mmol/kg dry weight (dw), and intra-assay coefficients of variation was $< 5\%$.

Western blotting

Approximately a 20-30 mg piece of frozen muscle was ground to powder and homogenised in 120 μl of ice cold lysis buffer (25 mM Tris/HCl [pH 7.4], 50 mM NaF, 100 mM NaCl, 5 mM EGTA, 1 mM EDTA, 10 mM Na-Pyrophosphatase, 1 mM Na_3VO_4 , 0.27 M sucrose, 1 % Triton X-100, 0.1 % 2-mercaptoethanol) and supplemented with a protease inhibitor tablet (Complete mini, Roche Applied Science, West Sussex, UK). Homogenates were centrifuged at 14,000 g for 10 min at 4°C and the supernatant was collected. The protein content of the supernatant was determined using a bicinchoninic acid assay (Sigma, UK). Each sample was diluted with an equal volume of 2X Laemmli buffer (National Diagnostics, USA) and boiled for 5-min at 100°C . For each blot, a standard and internal control was loaded along with 50-100 μg of protein from each sample and then separated in Tris-glycine running buffer (10 X Tris/Glycine, Geneflow Ltd, Staffordshire, UK) using self-cast 4% stacking and 10 % separating gels (National Diagnostics, USA). Gels were transferred semi-dry onto nitrocellulose membrane (Geneflow Ltd, Staffordshire, UK) for 2-h at 200 V and 45 mA per gel in

transfer buffers (anode 1; 0.3 M Tris, 20 % methanol, pH 10.4; anode 2; 0.25 M Tris, 20 % methanol, pH 10.4; cathode; 0.4 M 6-amino hexanoic acid, 20 % methanol, pH 7.6). After transfer, membranes were blocked for 1 h at room temperature in Tris-buffered saline (TBST: 0.19 M Tris pH 7.6, 1.3 M NaCl, 0.1 % Tween-20) with 5% non-fat milk. The membranes were then washed for 3 x 5 min in TBST before being incubated overnight at 4°C with phospho-specific anti-bodies for AMPK^{Thr172} and p38MAPK^{Thr180/Tyr182} (all from Cell Signaling, UK) as well as total protein of GAPDH (from Cell Signaling, UK) all at concentrations of 1:1000 in 1 X TBST. The next morning, membranes were washed for a further 3 x 5 min in TBST and subsequently incubated with anti-species horseradish peroxidase-conjugated secondary antibody (Bio-Rad or Dako, UK) for 1-h at room temperature. After a further 3 x 5 min washes in TBST, membranes were exposed in a chemiluminescence liquid (SuperSignal, Thermo Fisher Scientific, Rockford, IL, USA) for 5-min. Membranes were visualised using a Bio-Rad Chemi-doc system, and band densities were determined using Image Lab image-analysis software. All raw densitometry data were used for statistical analysis. However, because it is technically incorrect to compare densitometry data between gels (and hence, between subjects), for graphical purposes each subject's pre-exercise values was normalised to 1 (hence no error bars are shown for this time point) such that values at post-exercise and 3 h post-exercise are subsequently expressed as fold change relative to pre-exercise values. This approach has been used previously by us (Bartlett et al., 2012; 2013; Morton et al., 2009) and other researchers (Perry et al., 2010). GAPDH was used for normalisation to phosphorylated proteins where appropriate (Serpiello et al. 2012). We confirmed through a one-way analysis of variance (ANOVA) for repeated measures that there was no change in GAPDH at any time point measured.

Real time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RNA isolation and cDNA synthesis

Total RNA was isolated from one pass of muscle biopsy per time point (20-30 mg) using Trizol reagent (Invitrogen), according to the manufacturer's protocol. RNA quality and quantity were determined using Implen Nanophotometer (Implen, Munchen, Germany) and the RNA was stored at -80°C. cDNA was synthesised using random hexamers (Applied Biosystems) and Superscript III enzyme (Life Technologies), using manufacturer's protocol.

Gene expression analysis by RT-qPCR

Gene specific expression data was obtained using probes selected from Human Universal Probe Library (PGC-1 α – probe 13 and GAPDH – probe 60) (Roche Diagnostics) with custom designed primers (MWG Eurofins). Forward and reverse primers for PGC-1 α were as follows: 5'-TGAGAGGGCCAAGCAAAG-3' and 5'-ATAAATCACACGGCGCTCTT-3', respectively. One μ l of each sample were analysed in duplicates with negative controls using AB 7500 Real-Time Quantitative PCR instrument (Applied Biosystems) and Agilent Brilliant II qPCR Master Mix with Low ROX (Agilent Technologies). One microliter of cDNA, 500 nM of each primer and 200 nM of probe were used for each 20 μ l reaction. The following cycling parameters were used: 50°C for 2

minutes, initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/elongation at 60°C for 1 minute. Data was collected and analysed using AB SDS 1.43 Software (Applied Biosystems, Foster City, USA). Changes in mRNA content were calculated according to the $2^{-\Delta\Delta C_t}$ method where GAPDH was used as the housekeeping gene (Heid et al. 1996). Forward and reverse primers for GAPDH were as follows: 5'- GCTCTCTGCTCCTCCTGTTC-3' and 5'- ACGACCAAATCCGTTGACTC-3', respectively.

Venous blood samples and biochemical analysis

Blood samples were obtained pre-, during and post-exercise and drawn from a superficial vein in the antecubital crease of the forearm using standard venepuncture techniques (Vacutainers Systems, Becton, Dickinson). Samples were collected into vacutainers containing EDTA and lithium heparin and were stored on ice until centrifugation at 2,000 rpm for 15 min at 4°C. Following centrifugation, aliquots of plasma were stored at -80°C for later analysis. Samples were analysed for plasma glucose, lactate, glycerol and NEFA concentration using commercially available kits (Randox Laboratories, Antrim, UK).

Statistical analysis

The responses of blood metabolites, protein and mRNA contents to the laboratory-based soccer-specific training simulation were evaluated using a one-way analysis of variance (ANOVA) for repeated measures. The Least Significant Difference (LSD) post-hoc test for multiple comparisons was performed in order to examine the effect of time (*i.e.*, pre-, 20-min, 40-min, post-exercise and 3-h after exercise). Muscle glycogen analysis (pre- to post-exercise) was conducted via a paired samples t-test. Results are presented as means \pm standard deviation (SD). *P*-values < 0.05 were considered significant.

Results

Physiological responses to LSSTS

The physiological responses to the laboratory-based soccer-specific training simulation are presented in Table 3. The average intensity of the LSSTS was $55 \pm 6\%$ $\dot{V}O_{2\max}$ which corresponded to an average heart rate of $150 \pm 12 \text{ b}\cdot\text{min}^{-1}$ ($77 \pm 6\%$ HR_{\max}) and average RPE across the 60 min LSSTS of 6 ± 1 .

Blood metabolites

Blood glucose concentration did not change either during or following the LSSTS ($P = 0.218$). Compared with pre-exercise ($1.6 \pm 0.6 \text{ mmol}\cdot\text{L}^{-1}$), blood lactate significantly increased during (20-min, $4.6 \pm 2.1 \text{ mmol}\cdot\text{L}^{-1}$; 40-min, $5.0 \pm 2.5 \text{ mmol}\cdot\text{L}^{-1}$) and immediately post-exercise ($5.3 \pm 2.6 \text{ mmol}\cdot\text{L}^{-1}$) ($P < 0.001$) (Table 4). There were also significant changes in NEFA and glycerol following exercise. The concentration of NEFA was significantly higher at 40-min ($0.5 \pm 0.1 \text{ mmol}\cdot\text{L}^{-1}$), immediately post-exercise ($0.6 \pm 0.2 \text{ mmol}\cdot\text{L}^{-1}$) and 3 h post completion of the LSSTS ($0.8 \pm 0.2 \text{ mmol}\cdot\text{L}^{-1}$) compared with pre-exercise ($0.4 \pm 0.3 \text{ mmol}\cdot\text{L}^{-1}$) ($P < 0.001$). Glycerol concentration during (20-min, $69 \pm 28 \mu\text{mol}\cdot\text{L}^{-1}$; 40-min, $105 \pm 46 \mu\text{mol}\cdot\text{L}^{-1}$), immediately post-exercise ($145 \pm 54 \mu\text{mol}\cdot\text{L}^{-1}$) and 3 h post completion of the LSSTS ($64 \pm 22 \mu\text{mol}\cdot\text{L}^{-1}$) were also significantly higher than pre-exercise ($29 \pm 19 \mu\text{mol}\cdot\text{L}^{-1}$) ($P < 0.001$) (Table 4).

Muscle glycogen

There was a significant reduction in muscle glycogen following the laboratory-based soccer specific training when compared to pre-exercise muscle glycogen concentrations (Pre; $397 \pm 86 \text{ mmol/kg dw}$, Post; $344 \pm 64 \text{ mmol/kg dw}$; $P = 0.039$) (Figure 2).

Signaling responses to LSSTS

There was no change in the phosphorylation of AMPK (Post, 1.2 ± 0.2 ; 3h, 1.2 ± 0.3) or p38MAPK (Post, 1.4 ± 1.4 ; 3h, 1.3 ± 1.3) immediately post-exercise or 3 h post-exercise compared with pre-exercise ($P > 0.05$) (Figure 3A and 3B).

PGC-1 α mRNA

Although there was no change in PGC-1 α mRNA expression immediately post-exercise, there was a significant 5-fold increase 3 h post-exercise compared with pre- and post-exercise levels ($P = 0.009$) (Figure 4).

Discussion

The aim of the current study was to examine the acute whole body metabolic and molecular responses associated with mitochondrial biogenesis in human skeletal muscle following a laboratory simulated soccer-specific training protocol. We show novel data by demonstrating that laboratory simulated soccer-specific exercise increases the expression of PGC-1 α mRNA. There was, however, no change in phosphorylation of AMPK and p38MAPK, **when measured by western blot**, suggesting the increase in PGC-1 α mRNA expression **may possibly** be independent of AMPK and p38MAPK. These results imply that soccer-specific training can lead to beneficial **increases in PGC-1 α mRNA** in skeletal muscle though the upstream signalling events that coordinate this transcriptional response during soccer-specific training are unclear. As such these data may have implications for **better understanding the specific ‘muscle’ responses to** soccer-specific training protocols in the field.

In order to address our aim, we developed a novel laboratory simulated soccer-specific bout of training using activity profiles that mimics professional soccer players’ ‘in season’ habitual training. Although previous soccer-specific laboratory simulations have been designed to simulate soccer matchplay (Drust, Reilly, & Cable, 2000; Gregson, Drust, Batterham, & Cable, 2002), to the author’s knowledge, no previous attempt has been made to develop a laboratory-based simulation that represents soccer-specific training sessions completed by elite professional players. Indeed, the work-rates and the relevant duration of each discrete bout of activity employed in this protocol were similar to that observed in the field based session (Table 1). It is acknowledged that the total number of discrete movements was slightly different between the training session and simulation. This is a consequence of the treadmill used in the simulation and the transition time that the equipment takes to change speeds between different movements. We also recognise the difference in time spent between 60-80 % HR_{max} in our simulation (Table 2), which we propose is probably due to the omission of the small utility movements and technical and tactical elements inherent to soccer activity. Despite these limitations key physiological responses to soccer training, i.e. average HR, time spent above 80 % HR_{max} and s-RPE to the LSSTS were similar to those recorded in the actual training sessions of the elite professional players (Jeong et al. 2013) (Table 2). From a metabolic perspective, the increase in blood lactate at 20 minutes into the exercise bout (4.6 ± 2.1 mmol·L⁻¹) and increases in NEFA and glycerol at 20 min, 40 min and immediately post-exercise are similar to the patterns observed in matchplay (Bangsbo, 1994; Bangsbo, Nørregaard, & Thorsø, 1991; Krstrup et al., 2006). Collectively, the LSSTS would therefore appear to be a valid representation of the organisation of field based professional soccer training and as such makes it a suitable tool for the examination of the **metabolic** and molecular responses to this type of soccer-specific activity.

PGC-1 α is suggested to be the ‘master regulator’ of endurance training adaptation. In the current study we observed a 5-fold increase at 3 h post-exercise in the expression of PGC-1 α mRNA following 60-min of soccer-specific laboratory simulated activity. Although this time-course and magnitude of fold change is similar to that observed previously in human skeletal muscle in both running (Bartlett et al., 2012) and cycling exercise (Cochran, Little, Tarnopolsky, & Gibala, 2010; Gibala et al., 2009) this is the first study to examine PGC-1 α mRNA responses following soccer-specific activity. Such findings

may suggest that cumulative bouts of soccer training enhances the oxidative capacity of skeletal muscle and thus increases the capacity for both fat and carbohydrate utilisation during exercise (Pilegaard & Richter, 2008). Furthermore, the global effect observed in soccer-specific training such as small-sided games (Impellizzeri et al., 2006) may be partly mediated by the transient increases in expression of PGC-1 α mRNA, which, over time culminate in mitochondrial biogenesis (Egan et al., 2013; Perry et al., 2010). Engaging in regular soccer activity (60 min) is also becoming well recognised as a valid mode of exercise for improving aspects of human health and for decreasing the risk of developing lifestyle diseases (Krustrup, Christensen, et al., 2010b; Randers et al., 2010; 2012). Indeed, the increases in PGC-1 α mRNA may provide clues as to one of the potential mechanisms controlling improvements in oxidative capacity, lipid oxidation, insulin sensitivity, phenotype transformation and body composition that are characteristic following periods of soccer training (Krustrup, Aagaard, et al., 2010a).

Following the observation of increased PGC-1 α mRNA, we attempted to explore the upstream mechanisms that may be partly responsible. Interestingly, we observed no change in phosphorylation status of both AMPK and p38MAPK. This is to the authors knowledge the first study to demonstrate an increase in PGC-1 α mRNA expression independent of any changes in two of its upstream regulators, AMPK and p38MAPK. Firstly, the failure to highlight such a signalling cascade may be associated with the low average intensity of the soccer-specific laboratory training simulation (approximately $55 \pm 6 \dot{V}O_{2max}$) (Egan et al., 2010). Secondly, the extended recovery periods of low-intensity activity between the high-intensity activities may be too long in duration, therefore, allowing recovery of the cellular energy status (i.e. free AMP) resulting in a dampened AMPK signalling response. Thirdly, muscle glycogen is shown to be a regulator of AMPK activity (Philp, Hargreaves, & Baar, 2012). In considering the soccer-specific training simulation utilised just 14 % of pre-exercise muscle glycogen levels and that we have shown 30-45 % muscle glycogen utilisation to increase AMPK phosphorylation (Bartlett et al., 2012) it would appear the overall intensity and substrate utilisation isn't great enough to initiate AMPK signalling. In addition to finding no change in AMPK we also observed no alteration in phosphorylation status of p38MAPK. It is unclear as to why the exercise bout in the current study failed to activate p38MAPK but the relatively short periods of high-intensity activity may not be long enough to induce mechanical disturbances that would result in activation of p38MAPK. It is acknowledged that soccer training contains a small amount (approx. 4 %) of utility movements (backwards, sideways), **as well as eccentric muscle loading, heading, kicking and tackling**, however, due to the current LSSTS being performed on a motorised treadmill and performing these utility movements is a safety risk to the participant it is difficult to rule out that during actual field-based soccer training greater mechanical disturbances may occur within skeletal muscle due to the increased 'cutting' movements and technical actions. **With this in mind, it is possible that a Type II error occurred.** Whilst, both AMPK and p38MAPK demonstrated no change, one candidate that may be responsible for the increased transcriptional response of PGC-1 α is that of calcium (Ca²⁺) signalling. For example, it is well known that Ca²⁺ plays a crucial role in the contraction-relaxation cycle of skeletal muscle such that transient increases in intracellular Ca²⁺ leads to an increase in CaMK signalling (Chin, 2004). Furthermore, high-intensity contractions result in changes in the amplitude

and frequency of calcium oscillations that appear consistent with the discrete bouts of high-intensity activity in the current study (Baylor & Hollingworth, 2003). Taken together, it may be speculated that the high-intensity activities during the course of the 60 minute LSSTS may result in transient increases in intracellular Ca^{2+} that subsequently activate CaMKII leading to increased expression of PGC-1 α mRNA. As such, future studies should attempt to quantify activation of calcium related signalling in response to the typical activity profiles that are inherent to soccer-specific training.

Conclusion

In conclusion, we present data that furthers the understanding of the physiology of soccer. From a 'muscle' perspective, it has previously been unclear as to the molecular responses to soccer-specific training. These data demonstrate that a single 60 min session of soccer-specific training activates the expression of PGC-1 α mRNA in human skeletal muscle though this occurs independent of any change in AMPK and p38MAPK. This would suggest that there might be other signals, i.e. Ca^{2+} , involved in the activation and transcriptional response of human skeletal muscle. Moreover, the global effect of soccer-specific intermittent exercise on aerobic performance and human health may be partly mediated by adaptations associated with mitochondrial biogenesis in human skeletal muscle.

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Fig 1 Schematical representation of the 20 min 36 s LSSTS. This block of training was completed three times to form a 60-min simulated soccer training session.

Fig 2 Muscle glycogen changes pre- and post- the laboratory-based soccer-specific intermittent protocol. * Denotes significant difference compared to pre-exercise ($P < 0.05$).

Fig 3 PGC-1 α mRNA expression following the laboratory-based soccer-specific training simulation. * Denotes significantly different compared to pre- and post-exercise ($P < 0.05$).

Fig 4 Phosphorylation of *AMPK* (A) and *p38MAPK* (B) following the laboratory-based soccer-specific training simulation.

Table 1. The activity profiles employed in a laboratory-based soccer-specific training simulation and the actual training session observed. Adapted from Jeong et al. (2013).

	SP	Wk	Jg	HSR	Sp
<u>Training</u>					
Total time (%)	41	37	11	9	2
Total number for each movement	104	147	135	63	23
Average duration of each movement (s)	14	12	6	5	3
<u>Simulation</u>					
Total time (%)	41	37	11	9	2
Total number for each movement	78	84	51	48	18
Average duration of each movement (s)	14	12	6	5	3

SP; Static pause, Wk; Walking, Jg; Jogging, HSR; High Speed Running, Sp; Sprinting

Table 2. The physiological responses during the laboratory-based soccer-specific training simulation and the actual training session observed. * Denotes significant difference between groups ($P < 0.05$). Adapted from Jeong et al. (2013).

	Mean HR (b.min-1)	Mean % of HRmax	% of time spent in the HR zone (% of HRmax)						TL (AU)
			100-90	90-80	80-70	70-60	60-50	< 50	
Training (n=10)	137 ± 8	72 ± 3	0.4 ± 1	22 ± 12	41 ± 8 *	19 ± 3 *	15 ± 7	2 ± 2	365 ± 63
Simulation (n=10)	136 ± 10	71 ± 5	3 ± 3	21 ± 15	33 ± 10	26 ± 7	13 ± 8	4 ± 6	356 ± 64

HR; Heart rate, HRmax; Maximal heart rate, TL; Training load

Table 3. Physiological responses during the LSSTS.

Exercise Variable	Means \pm SD
HR ($\text{b}\cdot\text{min}^{-1}$)	150 ± 12
HR (% HR_{max})	77 ± 6
Minute ventilation ($\text{L}\cdot\text{min}^{-1}$)	65 ± 17
Oxygen consumption ($\text{L}\cdot\text{min}^{-1}$)	2.4 ± 0.6
$\dot{\text{V}}\text{O}_2$ ($\text{ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$)	32 ± 3
% $\dot{\text{V}}\text{O}_{2\text{max}}$ (%)	55 ± 6
Session-RPE (AU)	6 ± 1

HR-heart rate, $\dot{\text{V}}\text{O}_{2\text{max}}$ –maximal oxygen consumption, RPE-ratings of perceived exertion, RER-respiratory exchange ratio

Table 4 Responses of blood metabolites to the laboratory-based soccer-specific intermittent protocol. * Denotes significantly different to pre-exercise ($P < 0.05$).

	Pre	+20 min	+40 min	Post	3 h
Glucose (mmol.L ⁻¹)	5.29 ± 0.62	5.53 ± 0.30	5.59 ± 72	5.89 ± 0.66	5.03 ± 0.27
Lactate (mmol.L ⁻¹)	1.57 ± 0.57	4.64 ± 2.08 *	5.01 ± 2.46 *	5.30 ± 2.56 *	1.01 ± 0.27
NEFA (mmol.L ⁻¹)	0.45 ± 0.27	0.37 ± 10	0.44 ± 12	0.60 ± 0.22	0.79 ± 0.20 *
Glycerol (μmol.L ⁻¹)	29 ± 19	69 ± 28 *	105 ± 46 *	145 ± 54 *	64 ± 22 *

FIGURE 1.

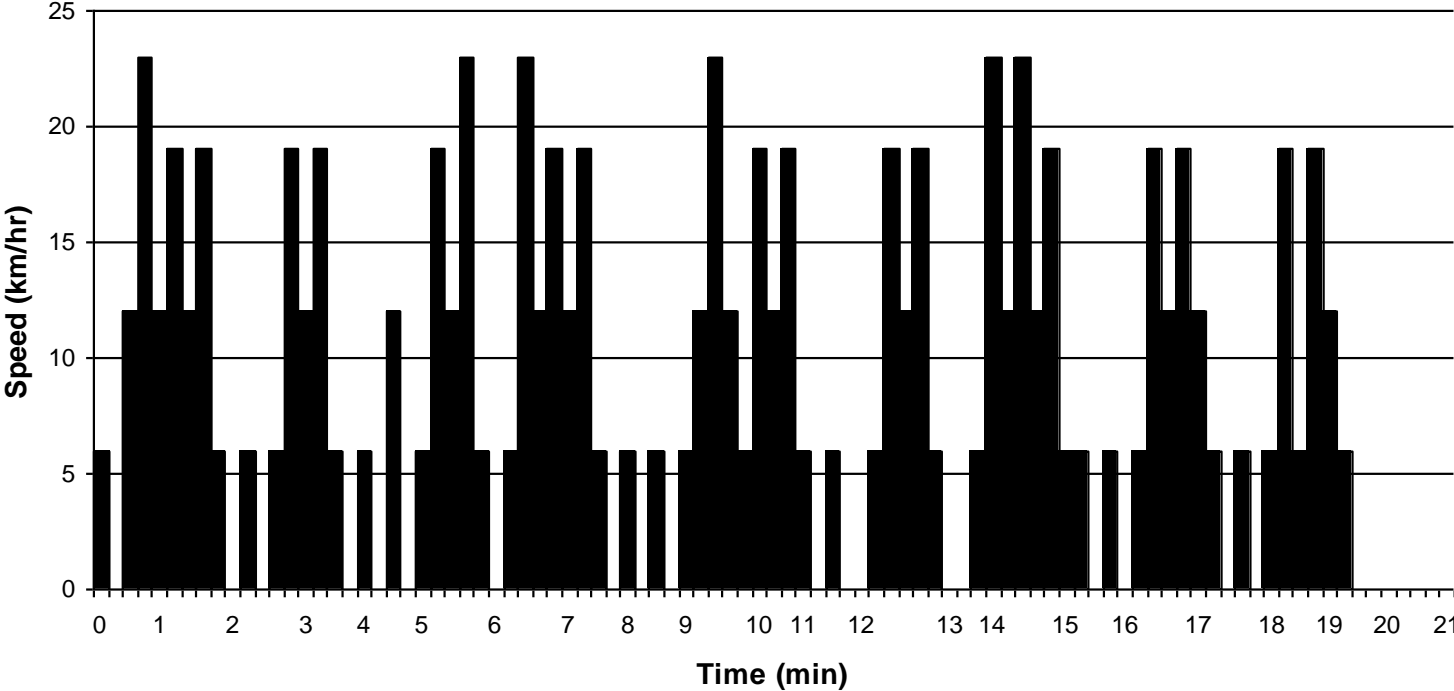


FIGURE 2.

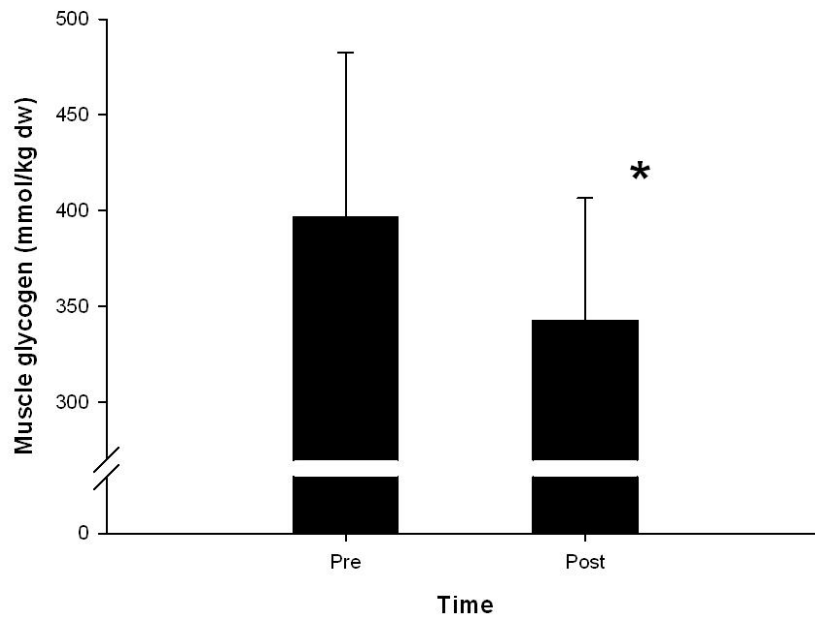
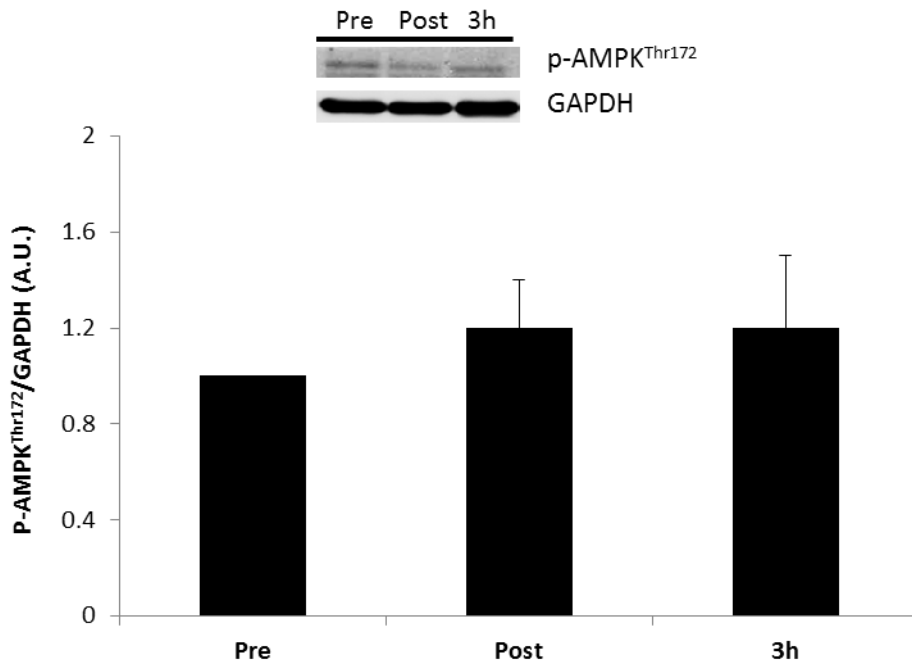


FIGURE 3.

(A)



(B)

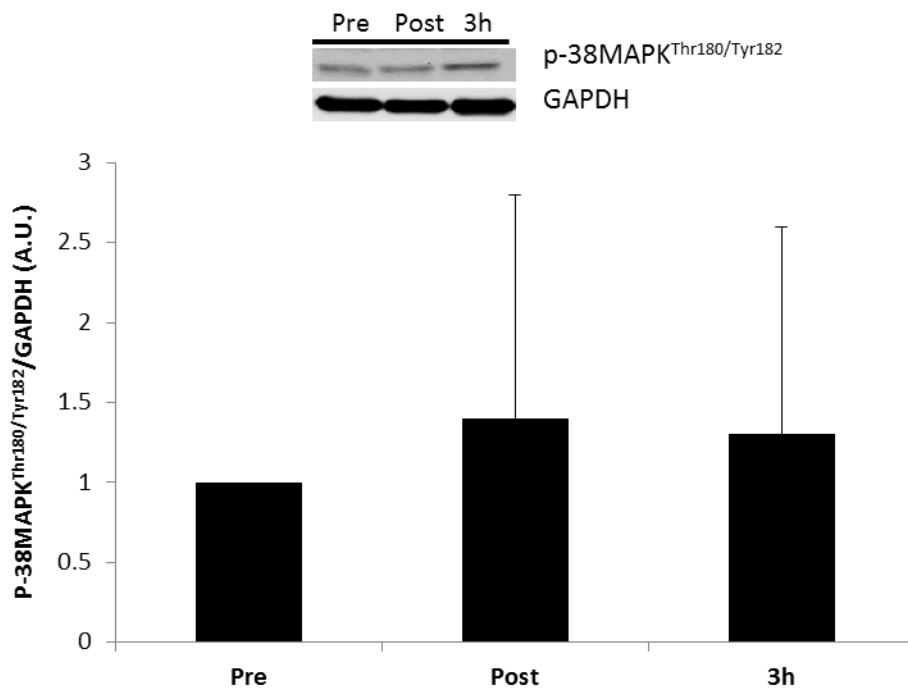


FIGURE 4.

