Promoter-specific regulation of *PPARGC1A* gene expression in human skeletal muscle

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Abstract

The goal of this study was to identify unknown transcription start sites of the *PPARGC1A* (*PGC-1α*) gene in human skeletal muscle and investigate the promoter-specific regulation of *PGC-1α* gene expression in human skeletal muscle. Ten amateur endurance-trained athletes performed high- and low-intensity exercise sessions (70 min, 70% or 50% $\dot{V}o_{2max}$). High-throughput RNA sequencing and exon–exon junction mapping were applied to analyse muscle samples obtained at rest and after exercise. *PGC-1α* promoter-specific expression and activation of regulators of PGC-1α gene expression (AMPK, p38 MAPK, CaMKII, PKA and CREB1) after exercise were evaluated using qPCR and western blot. Our study has demonstrated that during post-exercise recovery, human skeletal muscle expresses the *PGC-1α* gene via two promoters only. As previously described, the additional exon 7a that contains a stop codon was found in all samples. Importantly, only minor levels of other splice site variants were found (and not in all samples). Constitutive expression *PGC-1α* gene occurs via the canonical promoter, independent of exercise intensity and exercise-induced increase of $AMPK^{Thr172}$ phosphorylation level. Expression of *PGC-1α* gene via the alternative promoter is increased of two orders after exercise. This post-exercise expression is highly dependent on the intensity of exercise. There is an apparent association between expression via the alternative promoter and activation of CREB1.

Key Words
- skeletal muscle
- *PGC-1α*
- alternative promoter
- exercise

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Introduction

Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) is a key regulator of mitochondrial biogenesis, angiogenesis, as well as fat and carbohydrate metabolism in skeletal muscle (Olesen *et al*. 2010, Popov *et al*. 2015). Mouse and human skeletal muscle expresses several *PGC-1α* (*PPARGC1A*) gene isoforms originating from the canonical (*PGC-1α-a* mRNA) and alternative (*PGC-1α-b* and *PGC-1α-c* mRNA) promoters (Miura *et al*. 2008, Yoshioka *et al*. 2009). Alternative splicing at the intron between exons 6 and 7 can generate N-truncated (NT) PGC-1α isoforms (Zhang *et al*. 2009), which possess unique properties, different to those of

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full-length isoforms (Thom *et al*. 2014). Thus, the *PGC-1α* gene has the potential to produce at least six transcripts (Chang *et al*. 2012). However, another study in mouse skeletal muscle found only two previously described isoforms (*PGC-1α-a* and *NT-PGC-1α-b* mRNAs), as well as two additional isoforms (*PGC-1α-2* and *PGC-1α-3* mRNAs) with multiple splicing between exons (Ruas *et al*. 2012). Other tissue-specific promoters and isoforms have been described in human nervous tissue (Soyal *et al*. 2012) and liver (Felder *et al*. 2011).

Acute endurance exercise leads to increased *PGC-1α* gene expression in skeletal muscle. AMP-activated protein kinase (AMPK), p38 mitogen-activated protein kinase (p38 MAPK), and Ca^{2+}/calmodulin-dependent protein kinase (CaMKII) appear to be important for regulation of activity-induced *PGC-1α* gene expression from the canonical promoter (Zhang *et al*. 2014). Several groups (Norrbom *et al*. 2011, Ydfors *et al*. 2013, Popov *et al*. 2014) have shown that in human skeletal muscle, acute exercise induces *PGC-1α* gene expression, mainly via the alternative promoter. Based on studies in rodent skeletal muscle, it was proposed that activation of exercise-induced expression via the alternative promoter is regulated by the beta-2 adrenergic receptor-protein kinase A (PKA)-cAMP response element-binding protein (CREB1) signalling pathway (Chinsomboon *et al*. 2009, Tadaishi *et al*. 2011). Human myoblast (Norrbom *et al*. 2011) and mouse (Wen *et al*. 2014) studies showed that AMPK plays a role in the regulation of expression via the alternative promoter. However, another myoblast study (Yoshioka *et al*. 2009) and a study in isolated rat muscle (Tadaishi *et al*. 2011) did not confirm this finding.

Taken together, these findings indicate that alternative splicing of the *PGC-1α* gene has a strong influence on the properties of PGC-1α proteins, and that different signalling pathways activate the expression of the *PGC-1α* gene via different promoters. Investigations on the regulation of *PGC-1α* gene expression in human skeletal muscle have not been sufficient. The goal of this study was to identify unknown transcription start sites of the *PGC-1α* gene in human skeletal muscle, and to determine if mRNA splice site variants exist at rest and after acute exercise. For this purpose, high-throughput RNA sequencing and exon–exon junction mapping were applied to analyse samples obtained at rest and after exercise. The second goal was to investigate the promoter-specific regulation of *PGC-1α* gene expression in human skeletal muscle. For this purpose, we evaluated *PGC-1α* promoter-specific expression and activation of regulators of *PGC-1α* gene expression (AMPK, p38 MAPK, CaMKII, and CREB1) after aerobic exercise at different intensities (50 and 70% $\dot{V}o_{2max}$). *PGC-1a* mRNA expression in untrained subjects is higher after the first exercise session than after subsequent sessions (Perry *et al*. 2010). For this reason, the regulation of *PGC-1a* expression was investigated in skeletal muscle of subjects adapted to endurance training.

Materials and methods

Ethical approval

The study was approved by the Human Ethics Committee of the Institute of Biomedical Problems. The study complied with the guidelines set forth in the Declaration of Helsinki. All the participants provided their written consent to participate in this study.

Initial study

Ten amateur endurance-trained athletes (runners, cyclists, and cross country skiers with a median weight of 70 kg (interquartile range: 67–73 kg) and $\dot{V}o_{2max}$ of 61 ml/min per kg body weight (interquartile range: 58–62 ml/min per kg body weight)) participated in this study. During the first two visits to the laboratory, subjects were familiarized with the test procedures and completed an incremental ramp test on an Ergoselect 200 electromagnetic bicycle ergometer (Ergoline, Bitz, Germany). Initial load, load increment, and revolution rate were 0 W, 15 W/min, and 60–70 r.p.m. respectively. Each subject exercised until exhaustion. A revolution rate that slowed to 50 r.p.m. and a respiratory exchange ratio that increased to more than 1.1 were indicative of exhaustion. The pulmonary oxygen uptake rate ($\dot{V}o_2$) was measured at consecutive 15-s intervals, using an AMIS 2000 medical mass-spectrometer (Innovision, Odense, Denmark) with a mixing chamber. The highest $\dot{V}o_2$ value for 30 s was taken as $\dot{V}o_{2max}$.

Primary study

Each subject participated in high- and low-intensity exercise sessions (HE and LE), in random order, separated by 1 week. All subjects were instructed to refrain from vigorous aerobic and strength exercises for 1 week before the test, and from all exercise 36 h before. Subjects arrived at the laboratory at 0900 h and had a standardized breakfast (3624 kJ, 24 g protein, 157 g carbohydrate, and 15 g lipid). The cycling session started 1 h 45 min after breakfast, and consisted of a warm-up (5 min, 50% $\dot{V}o_{2max}$) and exercise session (65 min, 70% or 50% $\dot{V}o_{2max}$

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for HE and LE respectively). Two hours after the exercise session, the subjects had a standardized lunch (3714 kJ, 45 g protein, 183 g carbohydrate, and 27 g lipid). Capillary blood was drawn from the fingertip prior to, and at 20, 40, and 60 min after initiation of exercise. Lactate concentration was determined using a Super GL easy analyzer (Dr Mueller Geraetebau, Freital, Germany). The venous blood was drawn from the *v. intermedia cubiti* using catheter prior to, and at 30, 60, and 90 min after the exercise session. The plasma concentration of cortisol was evaluated using an ELISA-Cortisol kit (Immunotek, Moscow, Russia). Biopsies from the *m. vastus lateralis* were taken using the microbiopsy technique (Hayot *et al*. 2005) prior to exercise, and at 2 min, 4 h, and 8 h after under local anaesthesia (2 ml of 2% lidocaine). The muscle samples were quickly blotted with a gauze to remove superficial blood, frozen in liquid nitrogen, and stored at −80 °C until analysis. The first biopsy was taken 15 cm proximal to the *condylus lateralis ossis femoris*. Subsequent biopsies were taken 2 cm proximal to the previous one. The biopsy samples were taken on different days from different legs.

RNA extraction

Frozen samples (~20 mg) were sectioned at 20 μm, using a Leica ultratome (Leica Microsystems, Wetzlar, Germany). RNA was extracted from samples using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA concentration and purity were measured on a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, USA). After DNase treatment (Fermentas, Vilnius, Lithuania), an MMLV RT kit (Evrogen, Moscow, Russia) was used to obtain cDNAs with 1 μg of total RNA.

RNA-seq

Total RNA was taken from samples of two subjects for RNA sequencing prior to and at 4 and 8 h after exercise (six samples in total). The quality of RNA was checked for each sample by capillary electrophoresis, using an Agilent 2100 Bioanalyzer. All samples had an RNA integrity number >9. Library preparation was performed using the TruSeq RNA sample kit v2 (Illumina). Before sequencing, library concentration was assessed using a Qubit fluorimeter (Invitrogen) and by real-time PCR (primers: I-qPCR-1.1 AATGATACGGCGACCACCGAGAT and I-qPCR-2.1 CAAGCAGAAGACGGCATACGA). Libraries were diluted to 2300 h and sequenced on an Illumina HiSDefault 2000 instrument with a read length of 50 base pairs. Quality control and trimming were performed using CLC Genomics Workbench 6.5.1. High-quality reads were mapped to reference human genome GRCh37 and exon–exon junctions were identified using Tophat2 (v2.0.13) (mapping parameters: maximum of two mismatches, only uniquely mapped reads were allowed). Reads were counted for known exons of each gene using HTsDefault (v0.6.1). Total gene reads were considered as a measure of gene expression level. Exon–exon junction mappings and the total number of reads covering an exon–exon junction were visualized by the IGV tool (Thorvaldsdottir *et al*. 2013). To analyse differential gene expression, the R package DESeq method was used (Anders & Huber 2010).

Real-time PCR

Real-time PCR was performed using the Rotor-Gene Q cycler (Qiagen). The annealing temperature was optimized for each primer. The thermal profile included an initial heat-denaturing step at 95 °C for 5 min, followed by 45 cycles of denaturation at 95 °C for 15 s, annealing (56–60 °C) for 30 s, and extension at 72 °C for 30 s. Amplified genes were quantified by fluorescence using the EvaGreen master mix (Syntol, Moscow, Russia). The specificity of the amplification was monitored by analysis of melting curves and agarose gel (1%) electrophoresis. Each sample was run in triplicate, and a non-template control was included in each run. Target gene mRNA expression levels were calculated by the efficiency-corrected ΔCt method, as $(1+E_{ref})^{Ct\ ref}/(1+E_{tar})^{Ct\ tar}$, where *Ct* is the threshold cycle and *E* is the PCR efficiency. PCR efficiency was calculated using standard curves corresponding to target and reference genes (*RPLP0*, *GAPDH*). Expression of the *PGC-1α* gene from the canonical and alternative promoters was evaluated using primers aligned to exons 1a and 2, and exons 1b and 2 respectively. The total *PGC-1α* gene expression was evaluated using primer pairs to exon 2 (common for all transcripts). The primer sequences are shown in Table 1.

Western blot

Frozen samples (~10 mg) were sectioned at 20 μm, using a Leica ultratome (Leica Microsystems) and homogenized in ice-cold RIPA buffer containing protease and phosphatase inhibitors (50 mM β-glycerophosphatase, 50 mM NaF, 1 mM Na_3VO_4, 20 μg/ml aprotinin, 50 μg/ml leupeptin, 20 μg/ml pepstatin, and 1 mM PMSF). Samples were then centrifuged for 10 min at 10000×***g*** at 4 °C. Protein content was analysed by bicinchoninic acid (BCA) assay. Samples were mixed with Laemmli buffer (20 μg protein per lane),

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Table 1 Primers used in this study

Transcript	Strand	Sequence, 5′–3′	Product size (bp)
PGC-1α exon 1a-derived	Forward	ATGGAGTGACATCGAGTGTGCT	127
	Reverse	GAGTCCACCCAGAAAGCTGT	
PGC-1α exon 1b-derived	Forward	ATGGAGTGACATCGAGTGTGCT	156
	Reverse	AGTCCACCCAGAAAGCTGTCT	
Total PGC-1α	Forward	CAGCCTCTTTGCCCAGATCTT	101
	Reverse	TCACTGCACCACTTGAGTCCAC	
NR4A3	Forward	CATCGGTTTCGACGTCTCTTGT	125
	Reverse	CACTACGGCGTGCGAACCT	
MAFF	Forward	CCAAACCCTCCCAGGATTCAA	88
	Reverse	GGGGCATCTCTTCAGTGCAA	
SIK1	Forward	AGAGGACGGTGGAGTCACTG	102
	Reverse	CGCACTGGGCATTCCGATA	
RPLP0	Forward	CACTGAGATCAGGGACATGTTG	77
	Reverse	CTTCACATGGGGCAATGG	
GAPDH	Forward	CAAGGTCATCCATGACAACTTTG	496
	Reverse	GTCCACCACCCTGTTGCTGTAG	

PGC-1α, peroxisome proliferator-activated receptor gamma, coactivator 1 alpha; NR4A3, nuclear receptor subfamily 4, group A, member 3; MAFF, v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog F; SIK1, salt-inducible kinase 1; RPLP0, ribosomal protein, large, P0; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

loaded onto a 10% *T* polyacrylamide gel, and electrophoresis was performed in the Mini-PROTEAN Tetra cell system (Bio-Rad) at 20 mA per gel. The proteins were transferred onto nitrocellulose membranes using the Mini Trans-Blot system (Bio-Rad) in Towbin buffer for 3 h at 300 mA. Membranes were stained with Ponceau S to verify consistent loading of proteins, followed by washing and incubation in 5% non-fat dry milk for 1 h. Membranes were then incubated with anti-phospho-PKA Substrase (1:4000; Cell Signaling, Danvers, MA, USA), anti-phospho-AMP-Kα1/2^{Thr172} (1:200; Santa Cruz Biotechnology, Dallas, TX, USA), anti-phospho-ACC^{Ser222} (1:1000), anti-phospho-p38 MAPK$^{Thr180/Tyr182}$ (1:500), anti-phospho-CaMKII^{Thr286} (1:2500), anti-phospho-CREB1^{Ser133} (1:500), or anti-GAPDH (1:2500; all Abcam) overnight at 4 °C. The next day, membranes were incubated with anti-rabbit secondary antibody (Cell Signaling) for 1 h. After each step, membranes were washed with PBS-Tween 20 (three times for 5 min each). Finally, membranes were incubated with ECL substrate (Bio-Rad), and luminescent signals captured using a ChemiDoc Imaging System (Bio-Rad). All data are expressed as the ratio of phosphorylated protein to GAPDH.

Statistical analysis

Sample volumes were small with non-normal data distributions, and thus the data are expressed as median and interquartile range. The Wilcoxon matched pairs signed-rank test was used to compare repeated measurements. The relation between samples was evaluated by the Spearman rank correlation test. Level of significance was set at $P \leq 0.05$.

Results

Workloads for HE and LE were 3.2 (2.9–3.3) and 2.4 (2.2–2.5) W/kg body weight respectively. In contrast to LE, HE induced a pronounced metabolic response. Blood lactate level reached 4.3 (3.9–5.4) mM ($P<0.01$, initial level was 2.3 (1.8–2.6) mM) after the first 20 min of HE, and then did not change until termination of exercise (Fig. 1A). Blood cortisol level was elevated 1.4-fold after 30 min of termination of HE (from 295 (245–351) to 417 (364–567) nM, $P<0.05$, Fig. 1B)). On the other hand, both lactate level during LE and post-LE cortisol level demonstrated small decreases (from 1.9 (1.7–2.2) to 1.2 (1.1–1.6) mM, $P<0.01$ and from 335 (303–378) to 302 (208 to 327) nM, $P<0.01$, for lactate and cortisol respectively) and significantly differed ($P<0.01$) between HE and LE (Fig. 1A and B).

Exon–exon junction mapping showed that at rest, *PGC-1α* was expressed via the canonical promoter only (exon 1a-derived mRNA). HE induced expression from both the canonical and alternative promoter (exon 1b-derived mRNA) (Fig. 2A). The alternative promotor was located ~14 kb upstream of the canonical promoter, consistent with previous studies (Miura *et al.* 2008, Yoshioka *et al.* 2009). Exon 1c-derived mRNA was not detected in the post-exercise period. This observation is in agreement with our previous qPCR study, which showed very low post-exercise expression level of the exon 1c-derived *PGC-1α* mRNA in human skeletal muscle (Popov *et al.* 2014). No other transcription start sites were found (Fig. 2A). As previously described

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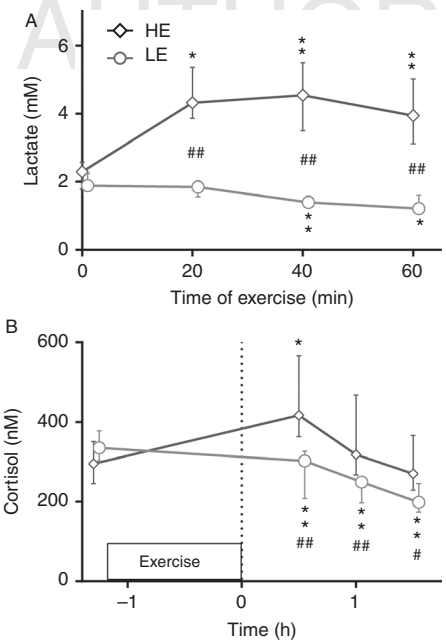

Figure 1
(A) The lactate in capillary blood before and during 70-min high- and low-intensity cycling bouts (70% and 50% $\dot{V}o_{2max}$ respectively). (B) The plasma cortisol level before and after exercise bouts. * and ** different from the initial level ($P<0.05$ and $P<0.01$, respectively); # and ## different between intensities ($P<0.05$ and $P<0.01$ respectively). Each value represents the median and interquartile range.

(Chang *et al*. 2012), the additional exon 7a that contains a stop codon was found in all samples (Fig. 2B). For other splice site variants, only low levels were detected (and not in all samples).

Quantitative PCR revealed that, at rest, *PGC-1α* expression from the canonical promoter was significantly higher than for the alternative promoter. The exercise-induced increase in *PGC-1α* expression via the canonical promoter was small (1.3-fold and $P<0.05$ for both LE and HE) and did not depend on exercise intensity (Fig. 3B). The expression of *PGC-1α-b* mRNA from the alternative promoter increased after both HE (471-fold, $P<0.01$) and LE (101-fold, $P<0.01$; Fig. 3C). Post-exercise *PGC-1α-b* mRNA expression was highly dependent on exercise intensity and reached a level threefold greater than for *PGC-1α-a* mRNA, i.e. the increase of expression *PGC-1a* via alternative promoters contributes to the increase of total *PGC-1a* mRNA to a greater extent than via canonical promoter. The total *PGC-1α* gene expression increased by 5.3- ($P<0.01$) and 2.3-fold ($P<0.05$) after HE and LE respectively (Fig. 3A).

Phosphorylation levels of p38 MAPK$^{Thr180/Tyr182}$ and CaMKII^{Thr286} did not change immediately after both LE and HE sessions (Fig. 4B and C). The phosphorylation level of AMPK^{Thr172} was increased only after HE (1.4-fold, $P<0.01$, Fig. 4D). The substrate for AMPK, ACC^{Ser222}, showed respective increases in phosphorylation levels (i.e. a marker of AMPK activity) of 4.0- and 2.8-fold, after HE and LE sessions ($P<0.05$ for both, Fig. 4E). The level of phospho-PKA substrates (i.e. a marker of PKA activity) did not change after both HE and LE (Fig. 4F). The CREB1^{Ser133} phosphorylation level increased (1.4-fold, $P<0.05$) after HE only. Post-exercise phosphorylation level was higher for HE ($P<0.05$) compared with LE (Fig. 4G).

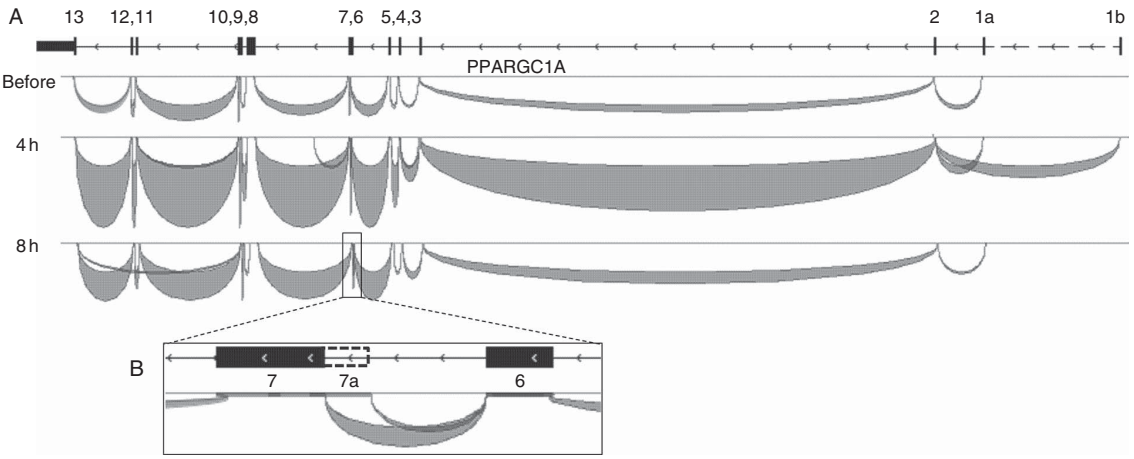

Figure 2
(A) Scheme of exons (vertical line) of the *PGC-1a* gene. Arches depict reads spanning an exon–exon boundary in *m. vastus lateralis* before, 4 and 8 h after acute high-intensity (70% $\dot{V}o_{2max}$) cycling bout. Thickness of each arch reflects amount of the reads. At rest, the *PGC-1α* is expressed via canonical promoter (exon 1a-derived mRNA) only. The exercise induces expression via both canonical and alternative (exon 1b-derived mRNA) promoters. (B) The additional exon 7a containing stop codon, which have been found in all samples. Only minor levels of other splice site variants (for instance, exons 10 to 13 junction at 8 h of recovery) were found (and not in all samples).

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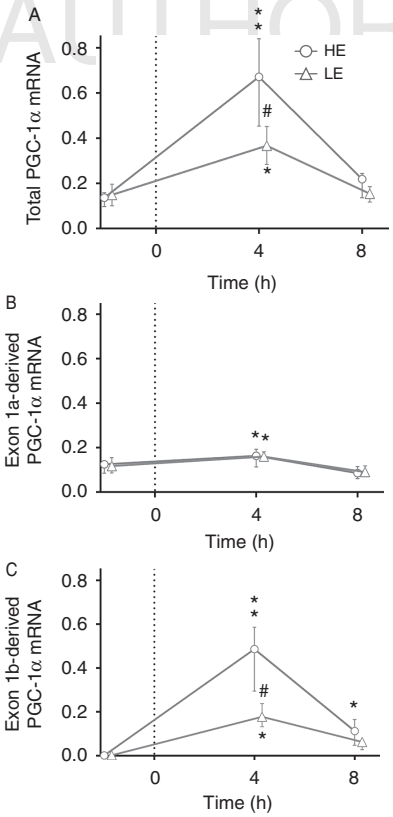

Figure 3
The total *PGC-1a* gene expression (A) and *PGC-1a* gene expression via (B) canonical (exon 1a-derived mRNA) and (C) alternative (exon 1b-derived mRNA) promoters in *m. vastus lateralis* before, 4 h and 8 h after acute high- and low-intensity cycling bouts (70% and 50% $\dot{V}O_{2max}$, respectively). 0 h – the termination of exercise. * and ** different from the initial level ($P<0.05$ and $P<0.01$ respectively); # different between intensities ($P<0.05$). Each value represents the median and interquartile range.

High-throughput RNA-seq analysis revealed an increase in CREB1-dependent gene expression at 4 and 8 h after termination of exercise. This was in contrast to another potential transcriptional regulator of *PGC-1α* gene expression that acts via the canonical promoter – myocyte enhancer factor 2 (MEF2C) (Fig. 5A). These findings were confirmed by qPCR (Fig. 5B, C, and D); the exercise-induced expression of CREB1-dependent genes *NR4A3*, *MAFF*, and *SIK1* at 4 h after termination of exercise was dependent on exercise intensity ($r=0.48$; $P=0.06$, $r=0.54$; $P<0.05$ and $r=0.6$; $P<0.05$ respectively). The comparison of mean gene expression data (*PGC-1a*, *NR4A3*, *MAFF*, and *SIK1*) with individual data of subjects 1 and 5 (samples involved in RNA sequencing) are shown in Supplementary Fig. 1, see section on supplementary data given at the end of this article.

The workload was correlated with expression of *PGC-1α-b* mRNA from the alternative promoter at 4 h after termination of exercise ($r=0.6$; $P<0.05$), as well as with post-exercise phosphorylation level of AMPK^{Thr172} ($r=0.79$; $P<0.001$). There was also a tendency toward significant correlation between the workload and phosphorylation level of CREB1^{Ser133} ($r=0.39$; $P=0.11$).

Discussion

Exon–exon junction mappings have confirmed that in human skeletal muscle, the *PGC-1α* gene is expressed via

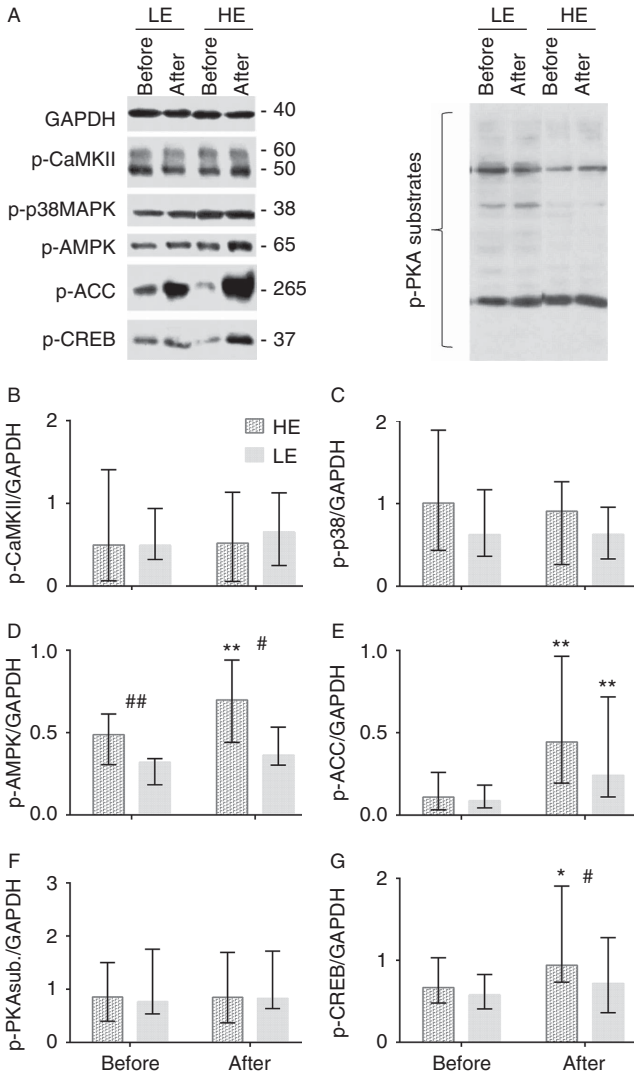

Figure 4
(A) The representative immunoblots and phosphorylation levels of (B) CaMKII^{Thr286}, (C) p38MAPK$^{Thr180/Tyr182}$, (D) AMPKα1/2^{Thr172}, (E) ACC^{Ser222}, (F) PKA substrates, (G) and CREB1^{Ser133} in *m. vastus lateralis* before and after acute high- and low-intensity cycling bouts (70% and 50% $\dot{V}O_{2max}$ respectively). 0 h – the termination of the exercise. * and ** different from the initial level ($P<0.05$ and $P<0.01$ respectively); # and ## different between intensities ($P<0.05$ and $P<0.01$, respectively). Each value represents the median and interquartile range.

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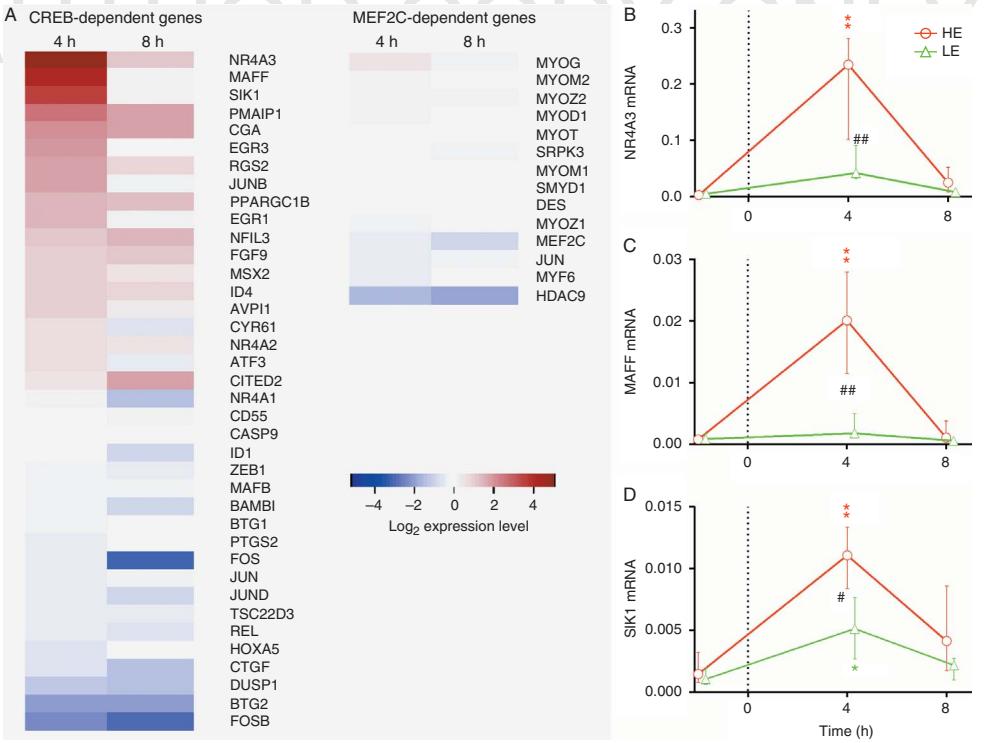

Figure 5

(A) Differential expression of CREB- and MEF2C-dependent genes in *m. vastus lateralis* before, 4 h and 8 h after acute high-intensity (70% $\dot{V}o_{2max}$) cycling bout. (B) The *NR4A3*, (C) *MAFF*, and (D) *SIK1* expression in *m. vastus lateralis* before, 4 h and 8 h after acute high- and low-intensity cycling bouts (70% and 50% $\dot{V}o_{2max}$ respectively). 0 h – the termination of exercise. * and ** different from the initial level ($P<0.05$ and $P<0.01$ respectively); # and ## different between intensities ($P<0.05$ and $P<0.01$, respectively). Each value represents the median and interquartile range.

the canonical and single alternative promoter, as described previously (Miura *et al.* 2008, Yoshioka *et al.* 2009). In addition to the *PGC-1α* mRNA that contains 13 exons, we have identified one major alternative splice site between exons 6 and 7. This splice site produces transcripts from both transcription start sites (*NT-PGC-1α-a* and *NT-PGC-1α-b* mRNAs) in human (Ydfors *et al.* 2013) and mice (Wen *et al.* 2014) skeletal muscle, and plays a significant role in *PGC-1α*-dependent regulation of angiogenesis (Thom *et al.* 2014). Importantly, only minor levels of other splice site variants were found (and not in all samples).

Multiple signaling pathways involved in contractile activity-mediated *PGC-1α* gene expression via canonical promoter, such as elevations in cytoplasmic calcium, AMPK and p38 MAPK activation were discussed in a previous study (Zhang *et al.* 2014). In a number of studies, activation of AMPK (Rasmussen & Winder 1997, Fujii *et al.* 2000, Chen *et al.* 2003, Sriwijitkamol *et al.* 2007, Egan *et al.* 2010), CaMKII (Rose *et al.* 2006, Egan *et al.* 2010), and stress-inducible p38 MAPK (Di Donato *et al.* 2014) positively associated with the intensity of exercise were shown in human skeletal muscle, while in one study (Egan *et al.* 2010) such association for p38 MAPK was not observed. We found that in endurance-trained human skeletal muscle, the canonical promoter *PGC-1α* is constitutively expressed at rest and after exercise. The exercise-induced elevation in expression via the canonical promoter is small (~30%), and was similar after low or high intensity exercise. Previously, a model of intensity-dependent regulation of *PGC-1α* gene expression, via the canonical promoter, had been proposed in rodent skeletal muscle (Tadaishi *et al.* 2011). According to this model, both high intensity exercise and pharmacological activation of AMPK increased expression via the canonical promoter (Tadaishi *et al.* 2011, Wen *et al.* 2014). In our study, an intensity-dependent increase in $AMPK^{Thr172}$ phosphorylation level had no association with *PGC-1α* gene expression via the canonical promotor. A possible explanation for this finding is that in endurance-trained human skeletal muscle, expression via the canonical promoter at rest is close to maximal level; exercise-induced

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increases in AMPK activity therefore have little effect on expression via the promoter. In our study, activation of AMPK after HE might be associated with high AMP/ATP ratio and/or low post-exercise muscle glycogen level (Richter & Ruderman 2009), both changes being a result either of higher intensity itself or recruitment of high-threshold fibres (Kristensen *et al*. 2015). Expression via the canonical promoter may also be regulated by the kinases p38 MAPK$^{Thr180/Tyr182}$ and CaMKII^{Thr286}, through activation of MEF2C (McGee & Hargreaves 2004) and CREB1-related protein, activating transcription factor 2 (ATF2) (Akimoto *et al*. 2005, Wright *et al*. 2007). Our study did not reveal exercise-induced changes in phosphorylation levels of p38 MAPK$^{Thr180/Tyr182}$ and CaMKII^{Thr286}. Lack of exercise-induced increase of p38 MAPK$^{Thr180/Tyr182}$ phosphorylation might be associated with fitness level of subjects. Indeed, the increase of post-exercise p38 MAPK$^{Thr180/Tyr182}$ phosphorylation level was blunted in skeletal muscle adapted to endurance training in comparison to untrained muscle exercising with the same relative intensity (Coffey *et al*. 2006). On the other hand, the activity and phosphorylation level of CaMKII^{Thr286} rapidly increased just after initiation of dynamic muscle contraction (Rose *et al*. 2006). It is possible, that CaMKII^{Thr286} phosphorylation level rapidly decreases just after termination of exercise, which partially explains the lack of exercise-induced increase of CaMKII^{Thr286} phosphorylation in the present study.

We found that expression of the *PGC-1α* gene via the alternative promoter was markedly increased after both LE and HE sessions; post-exercise expression level via this promoter was threefold higher than via the canonical promoter, and was closely associated with exercise intensity. These findings are in agreement with previous studies in mice (Tadaishi *et al*. 2011, Wen *et al*. 2014). Expression via the alternative promoter is associated with exercise-induced metabolic stress (Norrbom *et al*. 2011) and with activation of the β2-adrenergic receptor-cAMP-PKA-CREB1 pathway (Miura *et al*. 2008, Yoshioka *et al*. 2009, Chinsomboon *et al*. 2009, Tadaishi *et al*. 2011, Wen *et al*. 2014). An earlier study demonstrated endurance exercise intensity-dependent increase of cAMP level in rat skeletal muscle (Goldfarb *et al*. 1989), but another study found no changes in post-exercise cAMP level in skeletal muscle of rats acclimatized to treadmill running (Sheldon *et al*. 1993). We did not find increase of phosphorylation level of PKA substrates (i.e. a marker of PKA activity) after both HE and LE. CREB1 can increase transcriptional activity in response to diverse stimuli (Shaywitz & Greenberg 1999), including phosphorylation by AMPK (Thomson *et al*. 2008). Aminoimidazole carboxamide ribonucleotide (AICAR) treatment in human myoblasts (Norrbom *et al*. 2011) and mice (Wen *et al*. 2014) revealed a role for AMPK in regulation of expression via the alternative promoter. However, another myoblast study (Yoshioka *et al*. 2009) and investigations in isolated rat muscle (Tadaishi *et al*. 2011) did not confirm this finding. In our study, theoretically, both PKA and AMPK as well as other stimuli may have roles in intensity-dependent increases in CREB1^{Ser133} phosphorylation levels and expression of CREB1-dependent genes.

The *PGC-1α* canonical promoter region contains one conservative CRE-binding (palindromic) site for CREB1 and two binding sites for MEF2C (Esterbauer *et al*. 1999). Using high-throughput RNA-seq analysis, we found no changes in MEF2C-dependent gene expression. However, expression of CREB1-dependent genes was increased after high-intensity exercise session, which confirmed CREB1 activation. Moreover, the CREB1 target genes, *NR4A3*, *MAFF* and *SIK1*, showed expression that depended on intensity of exercise, similar to CREB1^{Ser133} phosphorylation levels. We speculate that, at rest, low concentrations of phosphorylated CREB1^{Ser133} are sufficient to induce near-maximal expression via the canonical promoter. Therefore, exercise-induced activation of CREB1 has a low impact on regulation of expression via the canonical promoter. The *PGC-1α* alternative promoter region has a conservative CRE-binding site with a single nucleotide substitution (variant site) (Tadaishi *et al*. 2011). The variant site is functional but has a lower affinity for transcription factors, compared with the palindromic site (Heckert *et al*. 1996). This probably explains why expression via the alternative promoter, in resting muscle, is very low. Thus, exercise intensity-dependent increases in CREB1^{Ser133} phosphorylation levels may be sufficient to stimulate expression via the alternative promoter.

A previous study showed that the variant CRE-binding site in promoter of human α subunit gene depends more heavily on the upstream elements than a promoter with palindromic CRE (Heckert *et al*. 1996). The muscle-specific transcription factors, MyoD and MRF4, transactivate the alternative promoter in *PGC-1α* gene in myoblasts through interaction with a proximal E-box motif (Yoshioka *et al*. 2009). It is possible that in our study these transcription factors play a role in the intensity-dependent regulation of expression via the alternative promoter. Moreover, we cannot ignore the possibility that regulation of transcription via the alternative promoter is sensitive to other CREB1-related transcription factors,

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as well as to CREB1-dependent coactivators and repressors (Altarejos & Montminy 2011).

In conclusion, this study has demonstrated that during post-exercise recovery, endurance-trained human skeletal muscle expresses the *PGC-1α* gene via two promoters only. Constitutive expression occurs via the canonical promoter, independent of exercise intensity and $AMPK^{Thr172}$ phosphorylation level. Expression of *PGC-1α-b* mRNA, via the alternative promoter, is markedly increased only after exercise and has a greater contribution to the increase of total *PGC-1α* mRNA compared to mRNA expression, via the canonical promoter. This post-exercise *PGC-1α-b* mRNA expression is highly dependent on the intensity of exercise. There is an apparent association between expression via the alternative promoter and activation of CREB1. Clarification of the molecular mechanisms underlying *PGC-1α* gene expression in human skeletal muscle may provide an opportunity to control the expression of different isoforms through varying exercise patterns, and/or through pharmacological interventions. These could be important strategies for optimization of endurance training as well as for treating patients with various diseases such as diabetes mellitus and metabolic syndrome.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JME-15-0150.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
D P and O V designed the studies. D P, E L, T V, and N K conducted the experiments. D P, E L, T V, N K, and P M analysed the results. D P, and O V wrote the manuscript and all authors approved the final version of the manuscript.

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