Alternative splice variant PGC-1α-b is strongly induced by exercise in human skeletal muscle

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Norrbom J, Sällstedt EK, Fischer H, Sundberg CJ, Rundqvist H, Gustafsson T. Alternative splice variant PGC-1α-b is strongly induced by exercise in human skeletal muscle. Am J Physiol Endocrinol Metab 301: E1092–E1098, 2011. First published August 23, 2011; doi:10.1152/ajpendo.00119.2011.—The present study investigated whether exercise induces the expression of PGC-1α splice variants in human skeletal muscle and the possible influence of metabolic perturbation on this response. The subjects exercised one leg for 45 min with restricted blood flow (R-leg), followed by 45 min of exercise using the other leg at the same absolute workload but with normal blood flow (NR-leg). This ischemic model (R-leg) has been shown previously to induce a greater metabolic perturbation and enhance the expression of PGC-1α beyond that observed in the NR-leg. Cultured human myotubes were used to test suggested exercise-induced regulatory stimuli of PGC-1α. We showed, for the first time, that transcripts from both the canonical promoter (PGC-1α-a) and the proposed upstream-located promoter (PGC-1α-b) are present in human skeletal muscle. Both transcripts were upregulated after exercise in the R-leg, but the fold change increase of PGC-1α-b was much greater than that of PGC-1α-a. No differences were observed between the two conditions regarding the marker for calcineurin activation, MCIP1, or p38 phosphorylation. AMPK phosphorylation increased to a greater extent in the R-leg, and AICAR stimulation of cultured human myotubes induced the expression of PGC-1α-a and PGC-1α-c. AICAR combined with norepinephrine yielded an additive effect on the PGC-1α-a expression only. Our results indicate clearly that exercise can activate an upstream promoter in humans and support AMPK as a major regulator of the gene. The expression of PGC-1α-a is not sufficient to explain the increased expression of PGC-1α-b.

INCREASED MITOCHONDRIAL AND CAPILLARY DENSITIES are central components of skeletal muscle remodeling in response to repetitive exercise (3, 16, 34). The transcription coactivator peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) is an important regulatory factor for the coordination of these events (18, 21, 28). For example, PGC-1α plays a crucial role in skeletal muscle metabolism, fiber hypertrophy, and vascular growth via coactivation of different transcription factors (3, 11, 21). We and others have established previously that a single bout of exercise/contractile activity is sufficient to elicit a significant increase in the expression of PGC-1α mRNA in humans, rats, and mice (2, 4, 17, 24, 26). Existence of an alternative PGC-1α splice variant in skeletal muscle was originally observed using Northern blotting from animals after exercise (4). In mice, it was demonstrated recently that the exercise-induced increase in the transcription of PGC-1α was almost exclusively due to an activation of an alternative promoter located upstream of the canonical promoter reported previously in exon 1 (6). Activation of this alternative promoter gave rise to two new splice variants termed PGC-1α-a and PGC-1α-c (3, 6, 23). It is not known whether similar splice variants and mechanisms exist in humans.

Several signaling pathways have been suggested to regulate the PGC-1 family. The calcium-regulated pathways, calcineurin, and calcium/calmodulin-dependent protein kinase (CaMK) signaling and its suggested downstream signaling factor p38 (37) enhance the transcription of PGC-1α (15, 19, 38). In recent years, AMP-activated protein kinase (AMPK) has evolved as the main regulator of PGC-1α in exercised muscles (8, 20, 33, 35, 40). Recently, activation of β2-adrenergic receptors was elegantly shown to be sufficient and crucial for the exercise-induced upregulation of the PGC-1α-b and PGC-1α-c splice variants (6, 23, 32). However, from a physiological point of view, it is well known that the adaptation to training is specific to the muscle performing the exercise. Therefore, the role of circulating hormones as the sole regulatory stimuli appears highly unlikely. Rather, skeletal muscle remodeling may occur via a concert of stimuli. For example, mitochondrial biogenesis is closely associated with metabolic perturbation. We have demonstrated previously that in humans the PGC-1α mRNA is upregulated to a greater extent in exercising muscle when oxygen delivery is reduced. This ischemia-mimicking condition enhances aerobic skeletal muscle remodeling, including angiogenesis and mitochondrial biogenesis (24).

The main aims of the present study were to test the hypothesis that, in human skeletal muscle, 1) exercise induces PGC-1α-b expression, 2) exercise increases the expression of PGC-1α-b to a greater extent than that of PGC-1α-a, and 3) the upregulation of these two splice variants is enhanced by metabolic perturbation. For the above purposes, we used an experimental human model of metabolic perturbation. An in vitro model of cultured human myotubes was used to test the exercise-induced regulatory stimuli suggested.

MATERIALS AND METHODS

Subjects. Twelve healthy male subjects were included in the study. Their mean (range) age, height, and weight were 24 (20–27) yr, 181 (173–190) cm, and 75 (63–90) kg, respectively, and their mean (range) maximal oxygen consumption (VO2max) was 5.1 (43–64) ml·kg−1·min−1. Well-trained subjects (VO2max >65 ml·kg−1·min−1) were excluded to maximize the subjects’ exercise responses (29). The study was approved by the Ethics Committee of Karolinska Institutet. All subjects gave their informed consent to participate in the study.

Experimental model. The method first described by Eiken and Bjurstedt (9) was used to restrict blood flow during exercise. The subjects performed a single one-legged knee extension exercise, using...
a pressure chamber model to induce blood flow restriction. The exercise session consisted of 45 min with restricted (R) blood flow to the working leg, followed by 45 min with normal, nonrestricted (NR) blood flow to the other leg. The subjects were randomized into two groups: one group exercised their right leg and the other group their left leg in the R-leg condition. The exercise session started with the R-leg condition, and each subject was instructed to exercise at the highest tolerable workload for 45 min, taking into account that the subject must complete the entire session. The workload profile was recorded. After a short rest, exercise in the NR-leg condition was performed using the same workload profile as that used in the R-leg condition but using normal atmospheric pressure. Accordingly, the two legs developed the same power and amount of work in each session, although the ischemic training was experienced as much more strenuous.

Muscle biopsies from the vastus lateralis muscle of both legs were obtained using the percutaneous needle biopsy technique at rest before each exercise bout (24 h after any previous exercise), followed by postexercise biopsies performed directly after and 2 h after exercise. The subjects were resting during this time. The prebiopsy in the NR-leg was performed at the same time as the 2-h postbiopsy in the R-leg. All biopsy samples were frozen within 10–15 s in liquid nitrogen and stored at −80°C until further analysis.

For cell culture purposes, part of a muscle biopsy (50 mg) obtained at rest was stored in sterile phosphate-buffered saline containing 1% penicillin-streptomycin at 4°C overnight before the extraction of cells. Extraction of cells from the biopsy sample was performed as described (5), with some modifications. In brief, the sample was washed, minced, and dissociated enzymatically in 5 ml of 0.25% trypsin-1 mM EDTA (all cell media were from Invitrogen, Stockholm, Sweden) at 37°C and 5% CO2 with gentle agitation for 20 min. Undigested tissue was allowed to settle for 5 min, and the supernatant was collected in a 15-ml conical tube. The cells were centrifuged at 1000 × g for 5 min, and the cell pellet was suspended in 4 ml of culture medium (Dulbecco’s modified Eagle’s medium (DMEM)-F-12 and 1% penicillin-streptomycin) containing 20% fetal calf serum (FCS). Digestion of the slurry was repeated twice. The cells were cultured in T75 flasks (Sarstedt, Stockholm, Sweden), and growth medium was changed every 3rd or 4th day until 60% confluence was reached. For the experiment, myoblasts were grown in growth medium containing 20% FCS. At 80% confluence, the medium was replaced with differentiating medium (DMEM-F-12 and 1% penicillin-streptomycin) containing 2% FCS. On day 5 of culture with differentiating medium, the cells were treated with either 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR; 1 mM), norepinephrine (NE), or a combination of AICAR and NE, or they were not treated (control) for 24 h.

RNA extraction and mRNA quantification. Total RNA from skeletal muscle biopsies was prepared using the acid phenol method (7) and quantified spectrophotometrically by measuring absorbance at 260 nm. RNA from primary skeletal muscle cells was extracted using TRIzol (Sigma, St. Louis, MO). The integrity of total RNA was determined using 1% agarose gel electrophoresis. Two micrograms of total RNA was reverse transcribed with SuperScript reverse transcriptase (Life Technologies), using random hexamer primers (Roche Diagnostics) in a total volume of 20 μl.

RT-PCR was performed using the forward primers for exons 1a and 1b and the reverse primers for exons 4 and 5 (for visualization, see Fig. 1). PCR amplification was performed as described previously (14). PCR products were run on 1.5% agarose gels (0.02 μl/ml ethidium bromide) and visualized in a UV transilluminator. The amplified PCR product from forward primer 1b and reverse primer 5 was verified by sequencing (KI Gene; Karolinska University Hospital, Stockholm, Sweden).

Real-time RT-PCR was used to measure mRNA expression [ABI-PRISM 7700 Sequence Detector and 7500 Fast Real-Time PCR system (for analysis of the alternative splice variants); Applied Biosystems, Foster City, CA]. For the alternative PGC-1α splice variants, primers were designed (based on sequence homology) toward regions that identify PGC-1α splice variants transcribed from the canonical and the proposed upstream-located promoter (exon 1a and 1b). “Total” PGC-1α expression was determined using primers from exons 3 and 4, which should encompass transcripts initiated from both promoters and be comparable with the TaqMan analysis (synthesized by Cybergene, Stockholm, Sweden). The primers were modified from the mouse primers published previously (6) using the NCBI genome database (Fig. 1) to correspond to the human genome sequence and designed to cover exon-exon boundaries. The volume used for the real-time RT-PCR reaction was 15 μl, including 5 μl of sample cDNA diluted 1:100, forward primer (final concentration 0.4 μM), reverse primer (final concentration 0.4 μM), and SYBR Green PCR Master Mix (4309155E; Applied Biosystems). GAPDH was used as an endogenous control (4352934E; Applied Biosystems). All quantification reactions were checked using a melting curve. Primer efficiency was tested via standard titration curves and did not differ between the primer pairs used.

Oligonucleotide primers and TaqMan probes for myocyte-enriched calcineurin-interacting protein 1 (MCIP1) were designed using Primer Express version 1.0 (Applied Biosystems). The primers and probes were designed to cover exon-exon boundaries (24). 18S rRNA was selected as an endogenous control to control potential variations in RNA loading or in efficiency of the amplification (4310893E; Applied Biosystems). Amplification mixes (25 μl) contained 5 μl of sample cDNA diluted 1:100 (for the 18S sample, the cDNA was diluted 1:2000) and TaqMan Universal PCR Master mix. Control experiments revealed approximately equal efficiencies over different starting template concentrations for target genes and endogenous control. The relative distribution of the transcripts of interest was measured for each individual; a cycle threshold (Ct) value was obtained by subtracting the GAPDH or 18S Ct values from the respective target Ct values. The expression of each target was then evaluated using 2−ΔΔCt (ΔΔCt method) (36).

Protein extraction and Western blotting. Muscle samples (~20 mg) were homogenized in a buffer containing 20 mM HEPES (pH 7.5), 0.2 mM EDTA (pH 7.4), 1.5 mM MgCl2, 100 mM NaCl, 1 mM Na3VO4, 2 mM dithiothreitol, and 0.4 mM phenylmethylsulfonyl fluoride. NaCl...
Statistical analysis. The statistical analyses on mRNA data were conducted on ratios of target/endogenous control; a ΔC_T value was obtained by subtracting the 18S or GAPDH C_T value from the corresponding target C_T value. The expression of each target was then determined using the 2^−ΔΔC_T, which provides the level of expression of the target gene relative to the level of expression of the housekeeping gene in each sample. To ensure normal distribution of the relative expression of target gene to housekeeping gene, logarithmic transformation was performed to the base 10. A two-way ANOVA for repeated measurements was used to evaluate the mRNA response to a single bout of exercise (preexercise and 2 h postexercise) in the two exercise conditions (R-leg and NR-leg). Differences were considered significant at P < 0.05. A single-factor ANOVA was used to identify changes in mRNA expression in the cell culture experiments. Planned comparison was used (i.e., post hoc test) to identify significant interactions or, when no interaction was found, to identify differences corresponding to significant main effects in the ANOVA models described above.

Because of the few subject samples available for protein measurements, nonparametric Friedman’s analysis of variance using ranks for multiple dependent samples was used to evaluate the effects of time (preexercise, 0 h postexercise, and 2 h postexercise) and exercise condition (R-leg and NR-leg) on protein phosphorylation. Differences were considered significant at P < 0.05. Data are presented as means ± SE unless stated otherwise.

RESULTS

PGC-1α transcripts and exercise. Forward primers located in exons 1a and 1b combined with reverse primers located in exons 4 and 5 detected two splice variants. The bands observed corresponded to the calculated amplicon sizes. Sequencing of the PCR products verified that the correct sequences were amplified. The observation that the preexercise expression of the PGC-1α-b splice variant was much lower than that of the PGC-1α-a variant is clear for PCR products run on an agarose gel (Fig. 2). This was also confirmed by the detection of much lower preexercise C_T values for PGC-1α-b, as assessed using
real-time PCR analysis. A single bout of knee extension exercise with restricted blood flow (R-leg) led to a sevenfold and >100-fold increase in the levels of the PGC-1α-a and PGC-1α-b mRNAs, respectively, at 2 h after exercise (P < 0.05; Fig. 3, A and B). No significant changes were detected for the PGC-1α-a or PGC-1α-b mRNAs in the NR-leg condition (not significant; average fold increase ± SE: PGC-1α-a, 0.69 ± 0.12; and PGC-1α-b, 11.9 ± 7.03, 2 h after exercise relative to before exercise).

The PGC-1α-b mRNA levels represented <1% of the total PGC-1α mRNA before exercise but constituted around 10% of the total PGC-1α transcripts 2 h after exercise in the R-leg condition (P < 0.01; data not shown). Total PGC-1α mRNA increased 2 h after exercise in the R-leg condition (P < 0.01; Fig. 3C), but no change was observed in the NR-leg condition. The relative increase in total PGC-1 mRNA and in the PGC-1α-a variant was similar.

Upstream regulators of PGC-1α. No differences were observed in the levels of MCIP1 mRNA expression before exercise. Moreover, MCIP1 levels increased in both exercise conditions 2 h after the exercise bout (P < 0.05; Fig. 4A).

There was no significant difference in p38 phosphorylation, which increased in response to a single bout of exercise. The activation was observed immediately after the completion of the exercise bout in both exercise conditions and returned to preexercise values 2 h after the end of the exercise (P < 0.05; Fig. 4B). AMPK phosphorylation increased in the R-leg condition 2 h after a single exercise bout (P < 0.05; Fig. 4C), whereas no change was observed in the NR-leg condition. A greater increase in phosphorylation was observed immediately after exercise with restricted blood flow in four out of five subjects. A significant interaction was identified; however, it was detected only when all time points were included, presumably because of the small number of subjects available.

Cell culture. Differentiated human primary skeletal muscle cells were treated with either AICAR, NE, or a combination of AICAR and NE, or they were not treated (control), for 24 h. AICAR and NE stimulation alone induced the expression of the PGC-1α-b mRNA, which was undetectable in the control condition (Fig. 5). A greater increase in PGC-1α-b mRNA was found when AICAR and NE stimulations were combined (P < 0.05 in both comparisons, Fig. 5). The expression of the
PGC-1α-a mRNA increased in response to AICAR treatment alone and to the combination of AICAR and NE (P < 0.05 in both comparisons; Fig. 5). Total PGC-1α mRNA expression also increased in response to AICAR treatment and to the combination of AICAR and NE (P < 0.05 in both comparisons; Fig. 5).

DISCUSSION

The main and novel findings of this study were that 1) transcripts from both the canonical promoter (PGC-1α-a) and the proposed upstream-located promoter (PGC-1α-b) were present in human skeletal muscle; 2) a single bout of restricted blood flow exercise increased both transcripts, but PGC-1α-b increased to a much greater extent; 3) stimulation of cultured human primary myotubes with AICAR increased the expression of PGC-1α-a and PGC-1α-b; and 4) AICAR combined with norepinephrine showed an additive effect on PGC-1α-b only.

Data showed for the first time the existence of an alternative PGC-1α splice variant in humans, consistent with the reported findings from mice (4, 6). The basal PGC-1α-b expression level was very low compared with basal levels of PGC-1α-a. However, the percentage increase induced by exercise was much higher for PGC-1α-b compared with PGC-1α-a. This suggests that exercise activates a promoter upstream of the canonical promoter. It might be argued that multiple promoters and the existence of splice variants allow for tissue-specific regulation of expression or that specific splice variants are expressed differentially in response to various types of stimuli.

Activated CaMK, calcineurin A, and p38 MAPK signaling cascades are reported regulators of PGC-1α expression in skeletal muscle (1, 13, 25, 27, 37–39). In the present study, MCIP1 mRNA was upregulated in both the R- and the NR-leg conditions after a single bout of exercise. This should be considered in relation to the levels of PGC-1α mRNA, which increased only in the R-leg condition. Furthermore, no differences were seen in the phosphorylation of p38, a suggested downstream signaling factor in the calcineurin pathway (37).

Taken together, these findings argue against calcineurin and p38 MAPK as candidates for the regulation of PGC-1α in active skeletal muscle. Notably, other studies also challenged the importance of Ca2+ signaling for the activation of PGC-1α in the exercising muscle (12, 25, 33). Recently, AMPK gained increasing importance as the major regulator of the PGC-1α transcriptional response to exercise in humans (4, 20, 35, 40). In contrast to MCIP1 and p38 phosphorylation, AMPK phosphorylation increased only in the R-leg condition in the present study. This is in accordance with the greater metabolic perturbation, e.g., increased levels of lactate and adenosine metabolites, that occurs in the presence of restricted blood flow to the exercising muscle. This finding renders the AMPK pathway a strong candidate for the regulation of the exercise-induced transcription of PGC-1α. To investigate this candidate further, cultured human satellite cells were treated with AICAR and/or norepinephrine. AICAR stimulation increased the mRNA expression of both the PGC-1α-a and PGC-1α-b splice variants.

In our view, this supports a role for AMPK in the exercise-induced upregulation of PGC-1α-b. Findings obtained from mice and rats (22, 23, 31) suggest that PGC-1α-b is dependent mainly on β2-adrenergic stimulation, without any known effects mediated through AMPK activation. Using a similar exercise model, we reported previously a two to three times larger increase in the levels of circulating catecholamines in the presence of restricted blood flow compared with nonrestricted blood flow (30). Because almost all tissues throughout the body are exposed to exercise-induced changes in the circulating levels of catecholamines, it is unlikely that β-adrenergic stimulation by itself can regulate muscle-specific remodeling. Rather, skeletal muscle adaptation, e.g., mitochondrial biogenesis and angiogenesis, is highly specific to the exercised skeletal muscle (10). Moreover, acute β-adrenergic stimulation does not alter PGC-1α in resting human skeletal muscle (31). Finally, in the present study, the resting biopsy in the NR-leg was obtained at the same time as the biopsy obtained 2 h postexercise in the R-leg. Thus, the tissue had been exposed to increased levels of catecholamines that were similar to those of the R-leg but showed no sign of increased expression of the splice variants. Our data suggest that the β-adrenergic signaling alone is not sufficient to increase PGC-1α-b in human skeletal muscle. If β-adrenergic stimulation is involved in the observed exercise-induced upregulation of PGC-1α splice variants, it needs to be combined with an additional regulatory mechanism that is presumably localized to the exercising muscle. Interestingly, we observed that in vitro norepinephrine stimulation increased the expression of PGC-1α-b, but most importantly, a significantly greater increase was observed when norepinephrine was combined with AICAR. In mice and rats, AICAR stimulates the expression of PGC-1α-b (31); however, this was shown to be associated with an increase in the circulating levels of catecholamines. A specific β2-agonist, clenbuterol, was used in these studies performed with mice and rats, since activation of the alternative promoter has been suggested to be mediated through this adrenergic receptor subtype. In the current study, norepinephrine was selected because it is one of the catecholamines that increase with exercise; hence, it may represent a more relevant stimulation from a physiological point of view. The present findings lend support to the importance of β-adrenergic stimulation in the
regulation of PGC-1α-b and provide an idea of how effects of circulating hormones could be directed toward exercising muscles.

In conclusion, transcripts from both the canonical promoter (PGC-1α-a) and the proposed upstream-located promoter (PGC-1α-b) are present in human skeletal muscle, and a single bout of exercise upregulated both transcripts; however, the percentage increase of PGC-1α-b was much greater. This indicates clearly that exercise can activate a promoter located upstream of the canonical promoter in humans. The findings from exercising muscle and cell culture experiments support a role for AMPK as a major regulator of transcripts from the canonical promoter and the involvement of β-adrenergic stimulation in combination with AMPK in the regulation of PGC-1α-b.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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