Hypoxic Induction of Vascular Endothelial Growth Factor (VEGF) and Angiogenesis in Muscle by Truncated Peroxisome Proliferator-activated Receptor γ Coactivator (PGC)-1α

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Background: Peroxisomal proliferator activator receptor γ coactivator (PGC)-1α induces both mitochondrial biogenesis and angiogenesis in skeletal muscle.

Results: Hypoxic induction of an alternative spliced form of PGC-1α induces only angiogenesis in skeletal muscle, and not mitochondrial biogenesis.

Conclusion: Alternative splicing of PGC-1α explains how PGC-1α achieves specific induction of angiogenesis during hypoxia.

Significance: The findings suggest novel ways to induce angiogenesis in muscle without simultaneously inducing mitochondrial biogenesis.

The transcriptional coactivator peroxisome proliferator-activator receptor γ coactivator (PGC)-1α is required for full hypoxic induction of vascular endothelial growth factor (VEGF) in skeletal muscle cells. Under normoxic conditions, PGC-1α also strongly induces mitochondrial biogenesis, but PGC-1α does not activate this program under hypoxic conditions. How this specificity is achieved is not known. We show here that hypoxia specifically induces alternatively spliced species encoding for truncated forms of PGC-1α: NT-PGC-1α and PGC-1α 4. NT-PGC-1α strongly induces VEGF expression, whereas having little effect on mitochondrial genes. Conditioned medium from cells expressing NT-PGC-1α robustly induces endothelial migration and tube formation, hallmarks of angiogenesis. Transgenic expression of PGC-1α in skeletal muscle in mice induces angiogenesis in vivo. Finally, knockdown of these PGC-1α isoforms and hypoxia-inducible factor-1α (HIF-1α) abrogates the induction of VEGF in response to hypoxia. NT-PGC-1α and/or PGC-1α 4 thus confer angiogenic specificity to the PGC-1α-mediated hypoxic response in skeletal muscle cells.

Skeletal muscle is uniquely adaptable to extracellular physiological cues. For example, endurance exercise triggers mitochondrial biogenesis and neovascularization, ultimately improving fatigue resistance; nerve stimulation controls fiber type composition and maintains pro-growth signals; and ischemia triggers complex pathways, including a robust response to hypoxia. The many molecular pathways that regulate these processes are only beginning to be understood.

The transcription coactivator PGC-1α 4 is induced by exercise, nerve stimulation, and hypoxia in skeletal muscle (1). It is regarded as a crucial regulator of oxidative metabolism in muscle (2). PGC-1α binds to and augments the activity of several transcription factors at the promoters of nuclear-encoded genes. Co-activation of nuclear respiratory factor (NRF)-1 and NRF-2 induces the expression of nuclear-encoded mitochondrial genes, including almost all genes involved in oxidative phosphorylation. At the same time, PGC-1α indirectly regulates mitochondrial DNA (mtDNA) replication and transcription via increased expression of mitochondrial transcription factor A (TFAM) and other nuclear-encoded factors. PGC-1α thus coordinates metabolic gene expression in both the nuclear and the mitochondrial genomes. Transgenic expression of PGC-1α in muscle induces functional mitochondrial biogenesis, leading to decreased muscle fatigue and increased running endurance of the mice (3, 4).

We recently showed that PGC-1α also dramatically induces angiogenesis, thereby coordinating oxygen/fuel delivery (via vessels) with their consumption (in mitochondria) (5, 6). PGC-1α expression is induced by hypoxia in muscle cells and is required for full hypoxic induction of angiogenic genes such as VEGF. PGC-1α induces VEGF by co-activating the transcription factor estrogen-related receptor α (ERRα) on a novel enhancer located in the first intron of the VEGF gene. Transgenic expression of PGC-1α in muscle induces dramatic neovascularization, and these same mice are protected from muscle ischemic injury (5). Conversely, deletion of PGC-1α in skeletal muscle prevents exercise-mediated angiogenesis (7).

The abbreviations used are: PGC-1α, peroxisome proliferator-activator receptor γ coactivator-1α; NRF, nuclear respiratory factor; HIF-1α, hypoxia-inducible factor-1α; ERRα, estrogen-related receptor α; HUVEC, human umbilical vein endothelial cells; qPCR, quantitative real-time PCR; FL, full-length; ad, adenovirus; Alt, alternatively spliced; NT, N-terminal; TCA, tricarboxylic acid.

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These findings, however, raised a perplexing question. Hypoxia leads to the induction of VEGF and other hypoxic genes, but does not lead to the induction of mitochondrial genes. If PGC-1α is induced by hypoxia, and if PGC-1α is a potent activator of mitochondrial genes, then why are these genes not induced during hypoxia? We thus hypothesized that a specific form of PGC-1α protein must confer this specificity during hypoxia. Zhang et al. (8) recently reported the existence in liver and brown fat of an alternatively spliced biologically active truncated isoform of PGC-1α, termed NT-PGC-1α. Alternative splicing of the exon 6/7 boundary leads to a nonsense splicing event, the encoding of a premature stop codon, and a protein product less than one-half the size of full-length PGC-1α. Ruas et al. (9) demonstrated that transcription of this splice variant is likely initiated from an alternative promoter, and named this alternative messenger mRNA PGC-1α4. Neither study reported on the effects of these isoforms on angiogenic programs. PGC-1α4 and NT-PGC-1α differ in their N-terminal 12 and 16 amino acids, respectively. The remaining 250 amino acids are identical between both proteins and retain the sequences necessary for binding to ERRα (10), but not those required for binding to NRF-1 and NRF-2 (11). We thus reasoned that NT-PGC-1α and PGC-1α4 would likely induce VEGF and angiogenic genes more robustly than mitochondrial genes. We further hypothesized that alternative splicing at the exon 6/7 boundary of PGC-1α may be specifically induced by hypoxia, thus conferring specificity to the PGC-1α hypoxic response.

**EXPERIMENTAL PROCEDURES**

*Mice and Cells*—All animal experiments were performed according to procedures approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee (IACUC). HIF-1α floxed mice were from The Jackson Laboratory (007568), and both the PGC-1α transgenic mice, and the PGC-1α KO mice were from Dr. Spiegelman. Isolation and culture of primary skeletal myocytes were performed on entire hindlimb muscle after collagenase/dispase digestion, as described previously (15). Primary myocytes were differentiated in DMEM (5% horse serum).

*Generation of ad-NT-PGC-1α*—A blunt-end NT-PGC-1α product was generated by PCR from FL-PGC-1α using forward primer consisting of first 32 bp of PGC-1α and reverse primer consisting of 23 bp of homology to the 5′ end of exon 6 plus an additional 13 bp coding for the additional Leu-Phe-Leu-STOP codon of PGC-1α. Oligonucleotides were generated by PCR of plasmids encoding each specific isoform of PGC-1α, including both NT-PGC-1α and PGC-1α4, by qPCR (Fig. 1A). qPCR of plasmids encoding each specific isoform demonstrated the specificity of the qPCR primers (Fig. 1B). To evaluate expression of these spliced forms in skeletal muscle, differentiated primary murine skeletal myotubes were used. We have shown previously that hypoxia induces PGC-1α in these cells (5), but those results did not evaluate which isoform of PGC-1α was induced. Primary myoblasts were thus made to differentiate in cell culture, and were then treated with 0.5% oxygen for 16 h, versus normoxic control. Activation of a hypoxic program was confirmed by measuring expression of VEGF, Glut1, and PDK1, genes well known to be induced by hypoxia (Fig. 1C). As shown in Fig. 1D, hypoxia induced the expression of alternatively spliced PGC-1α 8-fold. The expression of FL-PGC-1α, on the other hand, was unaltered by

**RESULTS**

*Alternative Splicing of PGC-1α Is Preferentially Induced by Hypoxia in Skeletal Muscle Cells*—Oligonucleotides were generated to amplify specifically exon 6/7 alternative splice forms of PGC-1α, including both NT-PGC-1α and PGC-1α4, by qPCR (Fig. 1A). qPCR of plasmids encoding each specific isoform demonstrated the specificity of the qPCR primers (Fig. 1B). To evaluate expression of these spliced forms in skeletal muscle, differentiated primary murine skeletal myotubes were used. We have shown previously that hypoxia induces PGC-1α in these cells (5), but those results did not evaluate which isoform of PGC-1α was induced. Primary myoblasts were thus made to differentiate in cell culture, and were then treated with 0.5% oxygen for 16 h, versus normoxic control. Activation of a hypoxic program was confirmed by measuring expression of VEGF, Glut1, and PDK1, genes well known to be induced by hypoxia (Fig. 1C). As shown in Fig. 1D, hypoxia induced the expression of alternatively spliced PGC-1α 8-fold. The expression of FL-PGC-1α, on the other hand, was unaltered by...
hypoxic treatment. Hypoxia thus preferentially induces alternatively spliced PGC-1α expression in skeletal muscle cells.

**NT-PGC-1α Only Weakly Induces Mitochondrial Genes in Skeletal Muscle Cells—**To evaluate the function of NT-PGC-1α in muscle cells, an adenovirus that expresses NT-PGC-1α was generated. This virus was then used to infect primary myotubes. To be sure that any effects are independent of full-length PGC-1α present in myotubes, primary myoblasts were isolated from PGC-1α−/− mice and made to differentiate in culture. Infection of these primary myotubes lacking endogenous PGC-1α led to robust expression of NT-PGC-1α, as measured by Western blotting (Fig. 2A). The effects of infection with adeno-NT-PGC-1α were next compared with those of adeno-FL-PGC-1α, versus GFP-only control. Multiplicities of infection of each adenovirus were chosen that achieved equivalent, and moderate, induction of FL and NT-PGC-1α (Fig. 2B). As shown in Fig. 2C, FL-PGC-1α led to robust induction of various nuclear genes encoding important components of the mitochondrion, including cytochromes (cyts), electron transport chain complex IV (cox5b), ATP synthase (Atp5a), the TCA cycle (cs), and mitochondrial replication and transcription (TFAM). In sharp contrast, NT-PGC-1α only marginally induced these same genes (Fig. 2C). Measurements by Western blotting of mitochondrial complex III and V proteins revealed similarly poor induction by NT-PGC-1α (Fig. 2D). NT-PGC-1α thus only weakly induces mitochondrial genes in skeletal myotubes in culture. Finally, to directly and sensitively test the effects of NT-PGC-1α on mitochondrial function, NT-PGC-1α was more strongly overexpressed in primary myotubes, and cellular respiration was measured, using a Seahorse extracellular flux analyzer. As shown in Fig. 2E, despite the marked 5-fold greater overexpression of NT-PGC-1α versus FL-PGC-1α in this experiment, oxygen consumption rate was only moderately induced by NT-PGC-1α, roughly only half as much as that induced by FL-PGC-1α.

**NT-PGC-1α Strongly Induces VEGF and a Pro-angiogenic Phenotype in Muscle Cells—**Infection of PGC-1α−/− myotubes with FL-PGC-1α led to a 3–4-fold induction of the canonical pro-angiogenic peptide VEGF (Fig. 3A), as we have shown previously (5). We have shown that the induction of VEGF occurs via coactivation of the nuclear receptor ERRα (5), and indeed, PGC-1α induces the expression of ERRα itself as well (Fig. 3A). Infection with ad-NT-PGC-1α achieved more than double the induction of VEGF that was seen with FL-PGC-1α (Fig. 3A). This marked induction of VEGF coincided with only minimal induction of mitochondrial genes (Fig. 2C). The ratio of induction of VEGF to cyscs by NT-PGC-1α is thus more than 5-fold higher than by FL-PGC-1α (Fig. 3B). NT-PGC-1α directly coactivates ERRα in GAL-luciferase assays (Fig. 3C), consistent with its ability to induce VEGF (5) and its known ability to bind peroxisome proliferator-activated receptor α (PPARα) (8).

Endothelial migration and tube formation are hallmarks of angiogenesis. As shown in Fig. 3D, conditioned medium from skeletal muscle cells that overexpress FL-PGC-1α led to a 3-fold induction in the migration of endothelial cells in a Transwell assay, as we have shown before (5). Overexpression of NT-PGC-1α more than quintupled that induction, achieving 15-fold induction of endothelial migration over baseline (Fig. 3D).
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FIGURE 2. NT-PGC-1α only weakly induces mitochondrial genes in skeletal muscle cells. A, Western blot showing protein expression of NT-PGC-1α 48 h after infection of PGC-1α−/− myotubes with adenovirus expressing NT-PGC-1α. B, adenoviral multiplicities of infection were chosen to achieve matched overexpression of NT-PGC-1α and FL-PGC-1α expression. C and D, the expression of the indicated mitochondrial genes (C) and proteins (D) is induced in primary murine PGC-1α−/− myotubes by adenovirus encoding FL-PGC-1α, but only weakly by NT-PGC-1α. E, oxygen consumption rate (OCR) in primary myotubes infected with adenovirus expressing full-length PGC-1α, NT-PGC-1α, and GFP control. Left, gene expression; right, basal and maximal oxygen consumption rate. *, p < 0.05 by Student’s t test; §, p < 0.05 versus PGC-1α.

The addition of soluble fms-like tyrosine kinase 1 (sFlt1), a potent inhibitor of VEGF, completely abrogated the increase in endothelial migration (Fig. 3D), indicating that NT-PGC-1α induces endothelial migration largely via the secretion of VEGF. Lastly, conditioned medium from myotubes expressing NT-PGC-1α strongly stimulated the formation of tubes by endothelial cells (Fig. 3E). Together, these data indicate that, as compared with FL-PGC-1α, NT-PGC-1α strongly favors the activation of a pro-angiogenic program over a pro-mitochondrial program in muscle cells.

PGC-1α4 Induces Angiogenesis in Vivo—Ruas et al. (9) recently generated transgenic mice that express PGC-1α4 in skeletal muscle, using the MEF2C enhancer/myogenin promoter. We thus used these transgenic mice to test whether an alternatively spliced, truncated form of PGC-1α induces VEGF and angiogenesis in vivo. Tibialis anterior muscles were isolated from 12-week-old animals, and total RNA was prepared, and gene expression measured by qPCR. As shown in Fig. 4A, transgenic expression of PGC-1α4 induced total PGC-1α expression ~5-fold. Despite this induction, the expression of mitochondrial genes was only marginally induced (Fig. 4B). On the other hand, VEGF expression was significantly induced in these animals (Fig. 4C). PGC-1α4 thus favors the activation of a pro-angiogenic program over a pro-mitochondrial program in intact muscle. Thin frozen sections from the tibialis anterior muscle were next stained with antibodies against CD31, an endothelial specific marker that identifies capillaries. As shown in Fig. 4B, capillary density, expressed as number of capillaries per high powered field, or as capillaries per myofiber, was doubled in transgenic animals. PGC-1α4 thus induces angiogenesis in vivo.

Alternatively Spliced PGC-1α Mediates Hypoxic Induction of VEGF in Muscle Cells—The transcription factor HIF-1α mediates a large part of the transcriptional response to hypoxia in many, if not most, cells (12). To separate effects mediated by PGC-1α from those mediated by HIF-1α, we generated cells that lack HIF-1 activity. Primary myoblasts were isolated from animals carrying floxed alleles of HIF-1α. The myoblasts were then infected with adenovirus encoding for the Cre recombinase, leading to inactivation of the HIF-1α locus. The cells were then stably infected with lentivirus expressing shRNA targeted against HIF-1β (ARNT (aryl hydrocarbon receptor nuclear translocator)), the obligate heterodimer of both HIF-1 and HIF-2 transcription factors. Finally, the cells were made to differentiate into myotubes. As shown in Fig. 5A, HIF-1α and HIF-1β expression was largely absent in these cells. Despite strongly reduced HIF-1 expression, however, exposing the cells to 0.2% oxygen still led to significant induction of VEGF (Fig. 5B). Hypoxic induction of VEGF can thus occur HIF-independently in these cells.

We have shown previously that PGC-1α is required for maximal induction of VEGF expression by hypoxia in muscle cells (5). The data presented here suggest that alternative spliced forms of PGC-1α may be responsible for this effect. Consistent with this notion, Alt-PGC-1α was induced by hypoxia in HIF−/− cells (Fig. 5C) as robustly as in wild type cells (Fig. 1). To test the role of these isoforms directly, lentivirus was generated that encodes for shRNA to target specifically the alternatively
spliced PGC-1α transcripts, while leaving the FL-PGC-1α transcript unaffected. The myoblasts were then made to differentiate into myotubes and exposed to 0.2% oxygen, versus normoxia control. As shown in Fig. 5D, sh-Alt-PGC-1α completely abrogated the induction of VEGF in response to hypoxia, whereas sh-scrambled control had no effect. Truncated forms
of PGC-1α thus mediate hypoxic induction of VEGF in skeletal muscle cells. The data also indicate that the remnant hypoxic induction was not dependent on low grade HIF activity (either HIF-1 or HIF-2) because it was abrogated by shRNA directed at PGC-1α.

**DISCUSSION**

We show here that N-truncated forms of PGC-1α, as compared with FL-PGC-1α, preferentially induce an angiogenic program over a mitochondrial program in skeletal muscle cells. PGC-1α induces mitochondrial genes in large part via the coactivation of NRF-1 and NRF-2. The region of PGC-1α that binds to NRF-1 and NRF-2 remains poorly defined, but almost certainly lies outside the peptide sequences retained in NT-PGC-1α and PGC-1α4 (8, 11). On the other hand, these PGC-1α isoforms retain the LXXLL motifs via which PGC-1α interacts with ERRα, and we show here that NT-PGC-1α can coactivate ERRα. We have shown previously that PGC-1α induces VEGF expression via coactivation of ERRα (5). We thus propose that the specificity of NT-PGC-1α and PGC-1α4 for the angiogenic program is achieved via specific binding to ERRα, but not NRFs (Fig. 6).

We also show here that alternatively spliced forms of PGC-1α are induced by hypoxia in muscle cells and that these isoforms mediate hypoxic induction of VEGF. It is important to note that the HIF-1 pathway also contributes to this induction. For this reason, experiments were conducted in the absence of HIF-1 activity (Fig. 4). Hypoxic induction of PGC-1α occurs
independently of HIF-1 activity, and the induction of VEGF by 
PGC-1α also occurs independently of HIF-1. FL-PGC-1α has 
been proposed by others to induce VEGF indirectly via HIF-1, 
by inducing mitochondrial respiration, leading to elevated con-
sumption of oxygen, local hypoxia, and HIF-1 activation (13).
Our data suggest that this HIF-dependent mechanism is not at 
play with truncated forms of PGC-1α because mitochondrial 
genes are minimally induced. Muscle cells thus appear to acti-
vate two entirely separate pathways in response to hypoxia.
In general, prolonged hypoxia tends to repress the expression 
of mitochondrial complexes in most cell types. Our findings 
explain why hypoxic induction of PGC-1α does not induce 
mitochondrial genes, but the findings do not provide a mecha-
nism for the active repression of these genes. The mechanisms 
remain incompletely understood and likely differ between cell 
types. PGC-1α isoforms are not known to repress transcrip-
tional activity, and it thus seems unlikely that they would be 
involved in this active suppression of oxidative phosphoryla-
tion genes.
NT-PGC-1α largely resides in the cytoplasm (14). A signifi-
cant amount of NT, however, must also be nuclear because 
NT-PGC-1α robustly coactivates ERRα and induces VEGF 
expression. It will thus be of interest to evaluate whether hypoxia alters cellular localization of NT-PGC-1α. Future studi-
ies will also investigate how the expression of alternatively spliced PGC-1α is induced by hypoxia. This could occur at a transcriptional level or during post-transcriptional splicing. We show here that the induction occurs independently of HIF-1 activity, possibly suggesting a post-transcriptional event, such as regulation of alternative splicing.

Cyclic AMP and PKA promote nuclear localization of NT-PGC-1α (14). We have shown previously that PGC-1α is required for exercise-induced angiogenesis and that this also occurs in part via cAMP signaling (7). Testing whether truncated forms of PGC-1α are the principal isoform that mediate exercise-induced angiogenesis will be of interest, but will likely require specific modification of the murine genome. Exercise-induced mitochondrial biogenesis (15).

Ruas et al. (9) have recently shown that PGC-1α4 regulates an IGF-dependent pro-growth program. It will be of interest to determine comprehensively which gene loci are occupied by NT-PGC-1α, PGC-1α4, and FL-PGC-1α. It will also be of interest to determine whether the pro-growth program remains active under hypoxic conditions.

In summary, the data presented here highlight unique features of truncated forms of PGC-1α in skeletal muscle cells and explain the paradoxical observation that PGC-1α appears to be dispensable for exercise-induced mitochondrial biogenesis (15).

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