PGC-1β regulates angiogenesis in skeletal muscle

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Rowe GC, Jang C, Patten IS, Arany Z. PGC-1β regulates angiogenesis in skeletal muscle. Am J Physiol Endocrinol Metab 301: E155–E163, 2011. First published March 1, 2011; doi:10.1152/ajpendo.00681.2010.—Aerobic metabolism requires oxygen and carbon sources brought to tissues via the vasculature. Metabolically active tissues such as skeletal muscle can regulate blood vessel density to match metabolic needs; however, the molecular cues that coordinate these processes remain poorly understood. Here we report that the transcriptional coactivator peroxisome proliferator-activated receptor-γ coactivator-1β (PGC-1β), a potent regulator of mitochondrial biology, induces angiogenesis in skeletal muscle. PGC-1β induces the expression of vascular endothelial growth factor (VEGF) in cell culture and in vivo. The induction of VEGF by PGC-1β requires coactivation of the orphan nuclear receptor estrogen-related receptor-α (ERRα) and is independent of the hypoxia-inducible factor (HIF) pathway. In coculture experiments, overexpression of PGC-1β in skeletal myotubes increases the migration of adjacent endothelial cells, and this depends on VEGF signaling. Transgenic expression of PGC-1β in skeletal myocytes dramatically increases muscular vessel density. Taken together, these data indicate that PGC-1β is a potent regulator of angiogenesis, thus providing a novel link between the regulations of oxidative metabolism and vascular density.

BLOOD VESSELS PROVIDE OXYGEN AND NUTRIENTS to tissues to meet their metabolic demands (6). This supply is maintained by the formation of new blood vessels from preexisting vessels in a highly dynamic and tightly regulated process termed angiogenesis. Angiogenesis can occur under both pathological conditions like tumor growth and physiological conditions such as embryonic development, pregnancy, and exercise (5, 7, 30, 33). The process is triggered by the secretion from tissues of numerous soluble factors, including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), angiopoietin (ANGPT), and fibroblast growth factor (FGF) (15). Highly metabolic and dynamic tissues such as skeletal muscle must be able to regulate the supply of nutrients to match the demand needed to perform work. However, the intrinsic signals within tissues that coordinate angiogenesis with the tissue’s metabolic needs remain poorly studied.

Hypoxia or low oxygen tension is one potent inducer of angiogenesis via activation of the hypoxia-induced factor (HIF) pathway (16). In the presence of sufficient oxygen, the HIF-1α transcription factor is hydroxylated on key prolines and degraded. However, under conditions of hypoxia, HIF-1α is stabilized and free to dimerize with HIF-1β and activate proangiogenic genes such as VEGF (32). This pathway is active in multiple forms of pathological angiogenesis, but its role in physiological angiogenesis is not clear. Mice that lack HIF-1α in skeletal muscle, for example, have more blood vessels in the deep portion of the muscle rather than fewer, as might be expected (23). Other pathways thus clearly exist.

The peroxisome proliferator-activated receptor (PPAR)γ coactivator 1 (PGC-1) family of transcriptional coactivators regulates metabolic function in various tissues (12, 19, 29). The PGC-1 family consists of three members: PGC-1α, PGC-1β, and PGC-related coactivator (1, 20). These transcriptional regulators have no intrinsic DNA-binding activity but instead are recruited to specific genes by binding to DNA-binding transcription factors. Many transcription factors are targeted by PGC-1s, including PPARs, estrogen-related receptors (ERRs), and nuclear respiratory factors, among many others (25, 40, 43). All three members of the PGC-1 family function in promoting a core program of mitochondrial function, including genes of fatty acid oxidation and transport, electron transport chain, and numerous other mitochondrial structural proteins (19). In addition, each coactivator has unique functions in different tissues, such as control of gluconeogenesis in liver by PGC-1α or macrophage differentiation by PGC-1β (13, 14, 36, 39, 45). Transgenic overexpression of either PGC-1α or -β specifically in skeletal muscle significantly increases mitochondrial content and renders the mice more capable of endurance exercise (3, 8, 21, 41).

Mitochondria require fuel and oxygen, which are transported to the cell via the vasculature. Therefore, mechanisms are likely in place to couple mitochondrial biogenesis to angiogenesis. Recently, we have shown that PGC-1α can increase angiogenesis in skeletal muscle in vivo (2). Conversely, deletion of PGC-1α within skeletal muscle resulted in an impaired angiogenic program in response to hindlimb ischemia and exercise (2, 9, 11, 17). PGC-1α is thus one important integrator of metabolic function and angiogenesis. Here we investigate the role of PGC-1β in angiogenesis in skeletal muscle. Using both cell culture and transgenic models we demonstrate that PGC-1β induces in myocytes a program of angiogenic factors that differs from that activated by PGC-1α and that PGC-1β in myocytes powerfully stimulates endothelial cell migration in cell culture and angiogenesis in vivo.

EXPERIMENTAL PROCEDURES

Animals. All animal experiments were performed according to procedures approved by the Beth Israel Deaconess Medical Center’s Institutional Animal Care and Use Committee. Unless otherwise indicated, 16-wk-old mice were used for all experiments.
MCK-PGC-1β transgenic mice (T37) have been described elsewhere (3).

Cells and reagents. All reagents were procured from Sigma, unless otherwise indicated. Immunostaining was performed using anti-CD31 antibody (BD PharMingen). Human umbilical cord endothelial cells (HUVECs) and C2C12 cells were maintained in endothelial basal medium-2 (EBM-2) and Dulbecco’s modified Eagle’s medium (DMEM; supplemented with 10% fetal bovine serum), respectively. Isolation and culture of primary skeletal myocytes were performed on entire hindlimb muscle after collagenase/dispase digestion, as described previously (24, 28). Primary myocytes were differentiated in DMEM (5% horse serum), and C2C12 cells were differentiated in DMEM (2% horse serum). Cells were infected with adenovirus at a multiplicity of infection of 10–30, and mRNA expression was measured 48 h later. The adenovirus expressing green fluorescent protein (GFP), PGC-1α, and PGC-1β has been described previously (35). Reporter plasmids containing VEGF enhancer, mutated enhancer, and concatemerized ERRs binding sites have been described (2).

Real-time PCR. Total RNA was isolated from mouse tissue and cultured cells using the TRizol (Invitrogen) and Turbocapture (Qiagen) method, respectively. Samples for real-time PCR analyses were reverse transcribed (Applied Biosystems), and quantitative real-time PCRs were performed on the cDNAs in the presence of fluorescent dye (SYBR green; Bio-Rad). Relative expression levels were determined using the comparative cycle threshold method (4).

Endothelial cell migration assay. Differentiated C2C12 myotubes in 24-well plates were infected with adenovirus expressing GFP or PGC-1β for 34 h. BSA or soluble Flt1 (100 ng/ml; R & D Systems) was added to the medium for 12 h. Then, 5 × 10⁶ cells of HUVECs were plated on the upper compartment of transwells (8.0 μm pore size) prewarmed with EBM-2 medium for 16 h at 37°C. HUVEC migration to the lower compartment of transwells was measured after 12 h. Migrated HUVECs were fixed with 4% paraformaldehyde in PBS for 20 min at RT, and cells that remained in the upper compartment were removed with cotton swabs. Cells were blocked with 5% BSA in PBS-Tween 20 (PBST; 0.2% Tween 20) and stained with phalloidin-FITC in PBST for 4 h to visualize filamentous actin. Transwell inserts were washed three times in PBST and mounted onto slides with DAPI mounting medium.

Histological analysis. Quantification of capillaries was performed computationally, using Volocity software (Improvision; Perkin Elmer), on three random fields chosen from the midportions of transverse sections from the indicated muscles. All quantifications were performed blindly.

Statistical analysis. The data are presented as means ± SE. Statistical analysis was performed with Student’s t-test for all in vitro experiments and ANOVAs for all in vivo experiments. P values of <0.05 were considered statistically significant.

RESULTS

PGC1β regulates VEGF both in vitro and in vivo. To investigate whether PGC1β induces an angiogenic program in skeletal myocytes, C2C12 myoblasts in cell culture were made to differentiate into myotubes and then infected with adenoviruses expressing PGC-1β vs. GFP control. Forty-eight hours later, RNA was isolated and subjected to reverse transcription, and the relative expressions of angiogenic genes were assessed by quantitative PCR (qPCR). PGC-1β overexpression led to a significant increase in VEGF-A expression (Fig. 1A). However, in contrast to PGC-1α (as a positive control), PGC-1β did not induce the expression of other angiogenic genes (ANGPT2 and PDGFB). C2C12 cells are immortalized cells that have lost many properties of true
myoblasts. To investigate the role of PGC-1\(\beta\) in primary cells, skeletal muscle myocytes were isolated from C57Bl6 mice and made to differentiate into myotubes in cell culture. Infection with adenoviruses expressing PGC-1\(\alpha\), PGC-1\(\beta\), and GFP led to results similar to those observed in C2C12 myotubes (Fig. 1B). In addition, both PGC-1\(\alpha\) and -\(\beta\) significantly inhibited the expression of basic FGF (bFGF).

To test whether PGC-1\(\beta\) induces VEGF in vivo, levels of VEGF-A message were measured in gastrocnemius muscle of either wild-type or transgenic mice overexpressing VEGF-A message. These mice have been described and induce PGC-1\(\beta\) expression in striated muscle (3). Similar to what was observed in cell culture, PGC-1\(\beta\) overexpression induced the expression of VEGF-A twofold, whereas PGC-1\(\beta\) did not induce the expression of ANGPT2 or PDGFB (Fig. 1C). Together, these data show that PGC-1\(\beta\) can induce a subset of the proangiogenic program activated by PGC-1\(\alpha\).

**Induction of VEGF by PGC-1\(\beta\) is HIF independent.** Induction of VEGF and angiogenesis has been studied most extensively in the context of hypoxia and the activation of the HIF-1 transcription factor pathway (32). Previously, we argued that PGC-1\(\alpha\) induces its angiogenic program independently of HIF-1\(\alpha\) activity (2), although others have suggested otherwise (27). Although PGC-1\(\beta\) shares moderate homology with PGC-1\(\alpha\), the repertoires of transcription factors coactivated by PGC-1\(\alpha\) and -\(\beta\) differ significantly (19, 29). Therefore, we sought to test whether PGC-1\(\beta\) requires the HIF pathways to induce the expression of VEGF-A. Mouse embryonic fibroblasts (MEFs) isolated from either wild-type or HIF-1\(\alpha\)\(-/-\) embryos were infected with adenoviruses expressing PGC-1\(\beta\) or GFP control. PGC-1\(\beta\) induced VEGF-A expression in these cells two- to threefold in both the absence and presence of HIF-1\(\alpha\) (Fig. 2A), suggesting that PGC-1\(\beta\) induces VEGF independently of HIF activity. It is possible, however, that some HIF activity remains in these cells due to the presence of HIF-2\(\alpha\). Moreover, MEFs are heterogeneous cells that poorly model intact tissue. To address both of these issues, primary satellite cells were isolated from mice homozygous for HIF-1\(\alpha\) floxed alleles and differentiated into myotubes in cell culture. The myotubes were then infected with adenovirus encoding Cre recombinase to delete the HIF-1\(\alpha\) alleles, as well as adenovirus encoding short hairpin against HIF-1\(\beta\) (also known as ARNT), vs. GFP control (Fig. 2B). HIF-1\(\alpha\) is the obligate heterodimer to both HIF-1\(\alpha\) and HIF-2\(\alpha\), and thus no HIF activity should be possible in the double-infected myotubes. To confirm the absence of HIF activity, the cells were treated with dimethyloxaloylglycine (DMOG; a hypoxia mimetic) for 24 h. As shown in Fig. 2C, induction of PDK1 and VEGF-A was also reduced at baseline in the double-infected cells. These cells were then infected with PGC-1\(\beta\) or control adenovirus for 48 h, and the expression of VEGF-A was then assessed by qPCR. As shown in Fig. 2D, PGC-1\(\beta\) induced VEGF-A two- to threefold in these cells whether they contained HIF activity or not. Therefore, PGC-1\(\beta\) induction of VEGF is HIF independent.

**ERR\(\alpha\)-dependent induction of VEGF by PGC-1\(\beta\).** Previously, we have shown that the induction of VEGF-A by...
PGC-1α required coactivation of ERRα (2). To test whether PGC-1β induction of VEGF was also an ERRα-dependent process, primary differentiated myotubes from wild-type or ERRα−/− cells were infected with adenoviruses encoding for PGC1-β vs. GFP control. Whereas PGC-1β induced VEGF-A fourfold in wild-type cells, PGC-1β failed to induce the expression of VEGF in the absence of ERRα (Fig. 3A). The repression of bFGF by PGC-1β was also blocked in ERRα−/− cells (Fig. 3C). The induction of other ERRα-dependent genes (cytochrome c, somatic) was also impaired, although interestingly, it was not completely blocked (Fig. 3D). Therefore, the induction of VEGF-A by PGC-1β is ERRα dependent.

Previously, we described a novel enhancer in the first intron of the VEGF-A gene that is responsive to PGC-1α (2). To test whether PGC-1α could activate this enhancer, a luciferase reporter plasmid containing the enhancer upstream of the SV40 promoter was cotransfected with plasmids expressing ERRα, PGC-1α or -β, or empty control vectors. Neither ERRα nor PGC-1α alone was sufficient to induce activity of the enhancer, but addition of both PGC-1β and ERRα synergized to activate the VEGF enhancer fourfold (Fig. 4A). Adding PGC-1α and -β together had no synergistic effect (data not shown). Sequence analysis of the VEGF enhancer revealed five putative ERRα binding sites (Fig. 4B); four of the five sites are highly conserved across vertebrate species. Systematic mutations of these conserved ERRα binding sites within the VEGF enhancer reporter construct resulted in a significant decrease in the ability of PGC-1β to activate the VEGF-A enhancer (Fig. 4B). Most notably, deletion of nucleotides 3,578–3,508 completely blocked the induction by PGC-1β (Fig. 4B). PGC-1β is known to interact with ERRα via amino acids located in exons 3–4. A mutant version of PGC-1β lacking these amino acids fails to interact with ERRα (37). This mutant had a significantly reduced ability to activate the VEGF-A enhancer (Fig. 4C). Taken together, these data indicate that PGC-1β activates expression of VEGF-A via coactivation of ERRα on conserved ERRα binding sites within the VEGF-A enhancer.

PGC-1β in myocytes promotes endothelial cell migration. The generation of new blood vessels requires the activation, proliferation, and migration of endothelial cells. Therefore, we tested whether PGC-1β expression in myocytes can stimulate the migration of adjacent endothelial cells. C2C12 cells were made to differentiate into myotubes in the bottom wells of modified Boyden chambers (Transwell system). The cells were then infected with PGC-1β or control virus. Thirty-four hours later, HUVECs were seeded into the top chamber of the Transwell system without the underlying cultured medium being changed. Twelve hours later, the endothelial cells that migrated to the bottom chamber were counted (Fig. 5A). As shown in Fig. 5, B and C, PGC-1β-infected C2C12 cells

![Image](http://ajpendo.physiology.org/)
markedly stimulated the migration of the endothelial cells compared with control-infected myotubes. Preincubation with soluble VEGF receptor (sFlt1), which binds to and inhibits VEGF function, completely abolished the induced migration. Overexpression of PGC-1α in C2C12 cells yielded similar results (data not shown). Taken together, these data show that PGC-1α in myocytes can increase the migration of adjacent endothelial cells at least in part via increased VEGF expression and secretion.

Increased angiogenesis in PGC-1β transgenic mice. To test whether PGC-1β can induce angiogenesis in intact organisms, the MCK-PGC-1β transgenic mice described above were used. Various skeletal muscles (quadriceps, tibialis anterior, and gastrocnemius) were harvested from the MCK-PGC-1β transgenics and littermate controls. Transverse sections were generated from the muscles and stained with antibodies against CD31 (PECAM), an endothelial-specific marker that highlights capillaries. As shown in Fig. 6A, transgenic expression of PGC-1β in skeletal myocytes leads to a dramatic induction of capillary density in skeletal muscle. There was an approximately fourfold increase in capillaries/mm² (Fig. 6B) and a twofold increase in capillaries per muscle fiber (Fig. 6C). The cross-sections of fibers of MCK-PGC-1β animals are smaller, as reported previously (3), explaining why the induction of the capillary/fiber ratio is less than that of capillaries/mm². The MCK-CRE transgene is activated in the late embryogenesis/early postnatal period, long after basic muscle development is complete; it is thus unlikely that the observed dramatic increases in vascular density are a developmental phenomenon. PGC-1β in skeletal myocytes thus powerfully induces angiogenesis in vivo.

DISCUSSION

We show here that the coactivator PGC-1β can drive robust angiogenesis in skeletal muscle in vivo. We cannot exclude the possibility that PGC-1β might also be increasing vasculogenesis, although this process is not thought to
occur in postnatal skeletal muscle. Although angiogenesis and vasculogenesis are fundamentally different processes, the net result would still be increased vascular density as observed. PGC-1α is well established as a powerful driver of mitochondrial biogenesis (3, 35, 38). Mitochondria require fuel and oxygen delivered via the vasculature. PGC-1α can thus coordinate the consumption of fuel and oxygen (mitochondria) with their delivery (blood vessels) in skeletal muscle.

PGC-1β likely induces angiogenesis in large part via the strong induction of VEGF, and this induction is independent of hypoxia and HIF activity. Angiogenesis has been thought to occur primarily in the context of hypoxia. Most common models of angiogenesis, including retinopathy of prematu-
Angiogenesis is a complex process, requiring the coordination of multiple cell types in space and time. The PGC-1 coactivators are known to coordinate large programs of gene expression in numerous contexts. They are thus also well suited to coordinate angiogenesis. Investigating how the PGC-1s coordinate this complex process is likely to lead to new insights into the mechanism of angiogenesis.

**GRANTS**

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**DISCLOSURES**

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