PGC-1α mediates exercise-induced skeletal muscle VEGF expression in mice

Lotte Leick,1 Ylva Hellsten,2 Joachim Fentz,2 Stine S. Lyngby,1 Jørgen F. P. Wojtaszewski,2 Juan Hidalgo,3 and Henriette Pilegaard1

1Centre of Inflammation and Metabolism and Copenhagen Muscle Research Centre, Department of Biology, Section of Molecular, Cellular, and Integrative Physiology; 2Copenhagen Muscle Research Centre, Molecular Physiology Group, Section of Human Physiology, Department of Exercise and Sport Sciences, University of Copenhagen, Copenhagen, Denmark; and 3Institute of Neurosciences and Department of Cellular Biology, Physiology, and Immunology, Autonomous University of Barcelona, Barcelona, Spain

Submitted 6 February 2009; accepted in final form 22 April 2009

Leick L, Hellsten Y, Fentz J, Lyngby SS, Wojtaszewski JF, Hidalgo J, Pilegaard H. PGC-1α mediates exercise-induced skeletal muscle VEGF expression in mice. Am J Physiol Endocrinol Metab 297: E92–E103, 2009.—The aim of the present study was to test the hypothesis that PGC-1α is required for exercise-induced VEGF expression in both young and old mice and that AMPK activation leads to increased VEGF expression through a PGC-1α-dependent mechanism. Whole body PGC-1α knockout (KO) and littermate wild-type (WT) mice were submitted to either 1) 5 wk of exercise training, 2) lifelong (from 2 to 13 mo of age) exercise training in activity wheel, 3) a single exercise bout, or 4) 4 wk of daily subcutaneous AICAR or saline injections. In skeletal muscle of PGC-1α KO mice, VEGF protein expression was ∼60–80% lower and the capillary-to-fiber ratio ∼20% lower than in WT. Basal VEGF mRNA expression was similar in WT and PGC-1α KO mice, but acute exercise and AICAR treatment increased the VEGF mRNA content in WT mice only. Exercise training of young mice increased skeletal muscle VEGF protein expression ∼50% in WT but with no effect in PGC-1α KO mice. Furthermore, a training-induced prevention of an age-associated decline in VEGF protein content was observed in WT but not in PGC-1α KO muscles. In addition, repeated AICAR treatments increased skeletal muscle VEGF protein expression ∼15% in WT but not in PGC-1α KO mice. This study shows that PGC-1α is essential for exercise-induced upregulation of skeletal muscle VEGF expression and for a training-induced prevention of an age-associated decline in VEGF protein content. Furthermore, the findings suggest an AMPK-mediated regulation of VEGF expression through PGC-1α.

Exercise training: angiogenesis; adenosine 5′-monophosphate-activated protein kinase; peroxisome proliferator-activated receptor-γ coactivator-1α; vascular endothelial growth factor

THE CAPILLARY BED IS ESSENTIAL for optimal oxygen and substrate exchange in skeletal muscle (28), and a training-induced increase in skeletal muscle capillarization is a well-known phenomenon and is associated with an improved oxidative capacity of the muscle (9). An age-dependent impairment of angiogenesis in response to ischemia has been demonstrated in rabbits (43, 44). However, the age-related impairment of angiogenesis is reversible by exercise (31).

Vascular endothelial growth factor (VEGF) is widely accepted as a critical factor in the angiogenic process, with VEGF stimulating vascular endothelial cell growth, survival, and proliferation (12). Thus, enhanced capillarization with exercise training correlates with increased expression of VEGF (2, 23). In accordance, decreased capillarization with advanced age is associated with lower VEGF expression and lower VEGF promoter activity, indicating a defect in transcriptional regulation of VEGF (11, 43, 44). In addition, inhibition of endogenous VEGF production dramatically reduces basal skeletal muscle capillarization (49), and angiogenesis induced by treadmill running in rodents has been demonstrated to be dependent on the availability of VEGF (2, 52). A single bout of dynamic exercise has been reported to be sufficient to induce VEGF mRNA and protein expression in skeletal muscle (10, 17, 18, 42), but the factors involved in regulating this response have not been clarified.

Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) is also robustly induced by exercise (6, 41) and is known to be a key regulator of mitochondrial biogenesis (32). PGC-1α is a transcriptional coactivator activating a broad range of transcription factors and thereby regulating transcription of their target genes (32). Recently, it was shown that PGC-1α effectively regulates VEGF possibly through the transcription factor estrogen-related receptor-α (ERRα) to elicit formation of new capillaries in rodent skeletal muscle (5). This novel PGC-1α/ERRα pathway was reported to be independent of hypoxia-inducible factor-1α (HIF-1α), which is a well-known hypoxia-induced transcription factor that regulates VEGF expression (4, 5).

Furthermore, it was demonstrated that ablation of the femoral artery induced an accelerated formation of new capillaries in mice overexpressing PGC-1α in skeletal muscle, whereas PGC-1α knockout (KO) mice had a markedly reduced ability to form new capillaries in the limb (5). These findings emphasize PGC-1α as a novel and important regulator of angiogenesis in skeletal muscle, but whether PGC-1α plays a role in regulating exercise-induced VEGF expression in both young and aged mice is unknown.

Exercise imposes a major metabolic challenge to the muscle, which increases the AMP/ATP ratio and activates the energy sensor 5′-AMP-activated protein kinase (AMPK) (54). Activation of AMPK by injection of mice with the AMPK activator 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) induces PGC-1α mRNA expression, and this effect is totally abolished in AMPKα2 KO mice, suggesting that the PGC-1α induction is mediated by AMPKα2 (27). Furthermore, repeated AICAR-induced activation of AMPK in mice increases PGC-1α expression, mitochondrial biogenesis (21, 26, 48, 55), VEGF protein expression, and angiogenesis in mouse skeletal muscle (38). Some of the effects of AMPK activation in skeletal muscle have been suggested to occur through PGC-1α.
(24), but whether AMPK-induced VEGF expression occurs through PGC-1α is unknown.

We hypothesized that PGC-1α is obligatory for exercise-induced upregulation of VEGF expression in both young and old mice. Furthermore, we hypothesized that AMPK activation by AICAR induces VEGF expression by a PGC-1α-dependent mechanism.

METHODS

Mice

PGC-1α KO on a C57BL/6 mouse strain was used in the experiments. Their generation and phenotype have been described elsewhere (30, 33), and this strain of PGC-1α KO mice has normal skeletal muscle fiber type composition compared with littermate wild-type (WT) mice. All mice were kept at an 11:13-h light-dark cycle and were used as control (no exercise) or experiment groups.

Table 1. Training study

<table>
<thead>
<tr>
<th>Age in mo (females)</th>
<th>Wheel running/day, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>~4</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>106±10</td>
</tr>
<tr>
<td>PGC-1α KO</td>
<td>93±13</td>
</tr>
<tr>
<td>~9</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>29±6</td>
</tr>
<tr>
<td>PGC-1α KO</td>
<td>31±7</td>
</tr>
<tr>
<td>~13</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>16±3</td>
</tr>
<tr>
<td>PGC-1α KO</td>
<td>14±4</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 8 for each group. Wheel running duration/day and body weight at 4–5 mo of age of female and male WT and PGC-1α KO mice in the training study (30). *Significantly different from PGC-1α KO mice within sex and group, P < 0.05.

Exercise Training

The muscle samples used in the exercise training part in the present study have previously been used in an other study (30). In short, PGC-1α KO and littermate WT mice were divided into either a training group (8 males and 8 females of each genotype) or a control group (8 males and 8 females of each genotype). The training groups completed 5 wk of treadmill exercise training consisting of 60 min at 14 m/min at a 10% slope 5 times/wk (Exer 4 treadmill; Columbus Instruments, Columbus, OH). All the mice used in the exercise training groups had access to an activity wheel in the cage (Minimitter activity cage). Time spent in activity wheel per day was recorded by a computer (Sigma Sport, Neustadt, Germany). To ensure similar running volumes in WT and PGC-1α KO mice, the activity wheel of some WT mice was blocked 4 h into the dark period every evening during the last 4 wk. Thus the wheel running duration per day was similar in WT and PGC-1α KO mice (30). Wheel running duration and body weight of WT and PGC-1α KO mice are shown in Table 1. All running wheels were blocked 36–37 h before mice were euthanized by cervical dislocation. White gastrocnemius (WG) and quadriiceps muscles were removed and quickly frozen in liquid nitrogen.

Aging and Lifelong Exercise Training

Female PGC-1α KO and littermate WT mice were divided into control (n = 8) and training groups (n = 8). Mice in the training group had access to an activity wheel (Minimitter activity cage) in the cage from 2 until 13 mo of age, whereas control mice were placed in similar cages but without running wheel. Time spent in activity wheel was measured as described above. To ensure similar running volumes in WT and PGC-1α KO mice, the activity wheel was blocked periodically for WT mice, and therefore, the wheel running duration per day was directed by the PGC-1α KO wheel. Wheel running duration and body weight of WT and PGC-1α KO mice are shown in Table 2. The mice were ~13 mo old when euthanized. All running wheels were blocked 36–37 h before mice were euthanized by cervical dislocation. WG and quadriiceps muscles were removed and quickly frozen in liquid nitrogen.

Table 2. Age study

<table>
<thead>
<tr>
<th>Age in mo (females)</th>
<th>Wheel running/day, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>~4</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>106±10</td>
</tr>
<tr>
<td>PGC-1α KO</td>
<td>93±13</td>
</tr>
<tr>
<td>~9</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>29±6</td>
</tr>
<tr>
<td>PGC-1α KO</td>
<td>31±7</td>
</tr>
<tr>
<td>~13</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>16±3</td>
</tr>
<tr>
<td>PGC-1α KO</td>
<td>14±4</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 8 for each group. Wheel running duration/day at ~4, ~9, and ~13 mo of age for female WT and PGC-1α KO mice in the age study. Body weight of female WT and PGC-1α KO mice at ~13 mo of age. *Significantly different from control mice within given genotype; †significantly different from PGC-1α KO mice within given group, P < 0.05.

Table 3. Single exercise study

<table>
<thead>
<tr>
<th>Body weight, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>WT</td>
</tr>
<tr>
<td>PGC-1α KO</td>
</tr>
<tr>
<td>Males</td>
</tr>
<tr>
<td>WT</td>
</tr>
<tr>
<td>PGC-1α KO</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 12–16 for each group. *Significantly different from PGC-1α KO mice within sex, P < 0.05.

Table 4. Single AICAR treatment study

<table>
<thead>
<tr>
<th>Body weight, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>WT</td>
</tr>
<tr>
<td>PGC-1α KO</td>
</tr>
<tr>
<td>Males</td>
</tr>
<tr>
<td>WT</td>
</tr>
<tr>
<td>PGC-1α KO</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 12–13 for each group. Body weight of female and male WT and PGC-1α KO mice in the single 5-aminoimidazole-4-carboxamide-1-ß-β-ribofuranoside (AICAR) study. *Significantly different from PGC-1α KO mice within sex, P < 0.05.
Table 5. Repeated AICAR treatment study

<table>
<thead>
<tr>
<th>Genotype (females)</th>
<th>Body Weight, g (Saline)</th>
<th>Body Weight, g (AICAR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>22.8 ± 0.1</td>
<td>22.8 ± 0.1</td>
</tr>
<tr>
<td>PGC-1α KO</td>
<td>20.0 ± 0.1</td>
<td>20.0 ± 0.1</td>
</tr>
</tbody>
</table>

Body weight of female WT and PGC-1α KO mice in the repeated AICAR treatment study. Values are means ± SE; n = 10 for each group.

Single Exercise Bout

Male and female PGC-1α KO and littermate WT mice either rested or performed a single 1-h exercise bout on a treadmill (14 m/min, 10% slope, Exer 4 treadmill; Columbus Instruments). Body weight of WT and PGC-1α KO mice is shown in Table 3. Female and male mice were divided equally between experimental groups and were euthanized by cervical dislocation either immediately after exercise or at 2 or 6 h after exercise, as described previously (30), and soleus, WG, and quadriceps muscles were removed and quickly frozen in liquid nitrogen.

Single AICAR Treatment

Male and female PGC-1α KO and littermate WT mice were given a single subcutaneous injection of AICAR (500 mg/kg body wt; Toronto Research Chemicals, Toronto, ON, Canada) dissolved in 0.9% NaCl or saline (0.9% NaCl), as described previously (26). Body weight of WT and PGC-1α KO mice is shown in Table 4. Female and male mice were divided equally between experimental groups, and mice were euthanized by cervical dislocation at 30 min or 4 h after the saline or AICAR injection. The quadriceps muscles were removed and quickly frozen in liquid nitrogen.

Repeated AICAR Treatment Protocol

Female PGC-1α KO and WT littermate mice were given daily subcutaneous injections of AICAR (500 mg/kg body wt; Toronto Research Chemicals) dissolved in 0.9% NaCl or saline (0.9% NaCl), as described previously (26). Body weight of WT and PGC-1α KO mice is shown in Table 5. AICAR was given over a 27-day period, with injection every 2nd day during the first 6 days and every day during the rest of the period.

Mice were anesthetized 24 h after the last AICAR injection by an intraperitoneal injection of pentobarbital sodium (6 mg of pentobarbital sodium/100 g body wt). The WG and quadriceps muscles were removed and quickly frozen in liquid nitrogen. All samples were kept at −80°C until further analysis.

RNA Isolation and Reverse Transcription

Soleus muscles from both legs were used, and portions of WG and quadriceps muscles (crushed in liquid nitrogen to ensure homogeneity) were used for RNA isolation. RNA isolation was performed on 15–20 mg of muscle tissue using a guanidinium thiocyanate-phenol-chloroform method, as described previously (40).

Reverse transcription (RT) was performed using the superscript II RNase H− system (Invitrogen, Carlsbad, CA), as described previously (40), and diluted in nuclease-free H2O.

PCR

Real-time PCR was performed using the ABI 7900 sequence detection system (Applied Biosystems, Foster City, CA). Primers and Taqman probes for amplifying gene-specific fragments were designed using mouse-specific database (Ensemble) and Primer Express (Applied Biosystems). Primers and probe were obtained from TAG (Toronto Research Chemicals) dissolved in 0.9% NaCl or saline (0.9% NaCl), as described previously (26). Body weight of WT and PGC-1α KO mice is shown in Table 3. Female and male mice were divided equally between experimental groups and were euthanized by cervical dislocation either immediately after exercise or at 2 or 6 h after exercise, as described previously (30), and soleus, WG, and quadriceps muscles were removed and quickly frozen in liquid nitrogen.

PCR

Real-time PCR was performed using the ABI 7900 sequence detection system (Applied Biosystems, Foster City, CA). Primers and Taqman probes for amplifying gene-specific fragments were designed using mouse-specific database (Ensemble) and Primer Express (Applied Biosystems). Primers and probe were obtained from TAG (Toronto Research Chemicals) dissolved in 0.9% NaCl or saline (0.9% NaCl), as described previously (26). Body weight of WT and PGC-1α KO mice is shown in Table 3. Female and male mice were divided equally between experimental groups and were euthanized by cervical dislocation either immediately after exercise or at 2 or 6 h after exercise, as described previously (30), and soleus, WG, and quadriceps muscles were removed and quickly frozen in liquid nitrogen.

Single Exercise Bout

Male and female PGC-1α KO and littermate WT mice either rested or performed a single 1-h exercise bout on a treadmill (14 m/min, 10% slope, Exer 4 treadmill; Columbus Instruments). Body weight of WT and PGC-1α KO mice is shown in Table 3. Female and male mice were divided equally between experimental groups and were euthanized by cervical dislocation either immediately after exercise or at 2 or 6 h after exercise, as described previously (30), and soleus, WG, and quadriceps muscles were removed and quickly frozen in liquid nitrogen.

Single AICAR Treatment

Male and female PGC-1α KO and littermate WT mice were given a single subcutaneous injection of AICAR (500 mg/kg body wt; Toronto Research Chemicals, Toronto, ON, Canada) dissolved in 0.9% NaCl or saline (0.9% NaCl), as described previously (26). Body weight of WT and PGC-1α KO mice is shown in Table 4. Female and male mice were divided equally between experimental groups, and mice were euthanized by cervical dislocation at 30 min or 4 h after the saline or AICAR injection. The quadriceps muscles were removed and quickly frozen in liquid nitrogen.

Repeated AICAR Treatment Protocol

Female PGC-1α KO and WT littermate mice were given daily subcutaneous injections of AICAR (500 mg/kg body wt; Toronto Research Chemicals) dissolved in 0.9% NaCl or saline (0.9% NaCl), as described previously (26). Body weight of WT and PGC-1α KO mice is shown in Table 5. AICAR was given over a 27-day period, with injection every 2nd day during the first 6 days and every day during the rest of the period.

Mice were anesthetized 24 h after the last AICAR injection by an intraperitoneal injection of pentobarbital sodium (6 mg of pentobarbital sodium/100 g body wt). The WG and quadriceps muscles were removed and quickly frozen in liquid nitrogen. All samples were kept at −80°C until further analysis.

Muscle Lysate Preparation

From soleus, WG, and quadriceps, muscle lysates were prepared by homogenization using a polytron (PT 1200; Kinematica). The homogenates were prepared as described previously (26). Homogenates rotated end over end at 4°C for 1 h. Lysates were prepared from the homogenates by centrifugation for 20 min at 16,000 g and 4°C. Total protein content was determined by the bicinchoninic acid method (Pierce Chemical). Unless stated specifically, all chemicals were of analytic grade from Sigma-Aldrich.

SDS-PAGE and Western Blotting

Muscle lysate proteins were separated using Tris·HCl gels (Bio-Rad) and transferred (semidry) to polyvinylidene difluoride membranes (Immobilon Transfer Membrane; Millipore). Standard Western blotting procedures were used for detection of specific proteins, as described previously (8). Following detection and quantification using a charge-coupled device image sensor and 1D software (Kodak Image Station, 2000 MM; Kodak), the protein content was expressed in arbitrary units relative to standard samples loaded in duplicates on each separate gel.

Primary antibodies used for Western blotting were phospho-AMPKα Thr172 no. 2535 (Cell Signaling Technology, Boston, MA)
ampK and AMPKα2 (kind donation from Prof. G. Hardie, Dundee, UK) detecting phospho-AMPKα Thr172 and AMPKα2 at ~63 kDa; phospho-p38 MAPK Thr180/Tyr182 no. 9211 and p38 MAPK no. 9212 (Cell Signaling Technology) detecting phospho-p38 MAPK Thr180/Tyr182 and p38 MAPK at ~42 kDa; hexokinase II (HKII) no. 2867 (Cell Signaling Technology) detecting HKII at ~100 kDa; VEGF (A-20), sc-152, no. J806 (Santa Cruz Biotechnology, Santa Cruz, CA), detecting VEGF protein at ~23 kDa; and CD31 (PCAM1, M-20), sc-1506, no. LO208 (Santa Cruz Biotechnology), detecting CD31 at ~130 kDa. Secondary antibodies used were anti-rabbit horseradish peroxidase-conjugated immunoglobulins (DakoCytomation, Glostrup, Denmark). AMPK and p38 MAPK phosphorylations were normalized to total AMPKα2 and total p38 MAPK protein content, respectively.

### Table 6. Capillary density, FCSA, capillary/fiber ratio, VEGF, and CD31 protein content in EDL muscle in WT and PGC-1α KO mice

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>PGC-1α KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capillary density</td>
<td>850±18.9</td>
<td>858±18</td>
</tr>
<tr>
<td>FCSA, mm²</td>
<td>1.53±0.05*</td>
<td>1.15±0.02</td>
</tr>
<tr>
<td>Capillary/fiber ratio</td>
<td>1.27±0.03*</td>
<td>0.98±0.01</td>
</tr>
<tr>
<td>VEGF protein, AU</td>
<td>2.42±0.6*</td>
<td>0.81±0.02</td>
</tr>
<tr>
<td>CD31 protein, AU</td>
<td>0.53±0.05</td>
<td>0.52±0.05</td>
</tr>
</tbody>
</table>

*Significantly different from PGC-1α KO mice within given group, \( P < 0.05 \).

Values are means ± SE; \( n = 7–10 \). FCSA, fiber cross-sectional area; EDL, extensor digitorum longus. Protein content is expressed as arbitrary units (AU) normalized to a control sample.

Histochemical Staining of Capillaries

The number of capillaries in muscle was determined on 8-μm transverse sections of frozen samples of the extensor digitorum longus (EDL) muscle. The sections were air-dried for 20 min and then fixed in −20°C acetone for 30 s. The sections were then again air-dried, FITC-conjugated Griffonia simplifolia (RL-1102, 1:100 vol/vol; Vector) was added, and the sections were incubated for 1 h. After addition of the lectin, all procedures were conducted in the dark. The sections were carefully rinsed with phosphate-buffered saline followed by distilled water, and the sections were mounted. Stained capillaries were examined and photographed in a Zeiss Axioplan microscope.

For each muscle sample, all analyses were performed on a minimum of three different areas. The origin of all pictures was blinded for the observer before quantification of fibers and capillaries. A counting frame was placed over each picture to allow quantification of capillary densities (CD) and capillary-to-fiber ratios (C:F), and the number of muscle fibers and capillaries was counted according to Gundersen’s rule A (16), whereby all structures of interest intersected by the upper and right edges of each particular square border were included and those intersected by the lower and left edges were excluded. C:F was defined as the total number of capillaries per total number of fibers, and CD was defined as the number of capillaries per mm². Mean fiber...
cross-sectional area (FCSA) was estimated as total area counted relative to the total number of fibers counted.

Statistics

All data are presented as means ± SE. Two-way analysis of variance was applied to evaluate the effect of genotype and exercise/AICAR treatment on body weight, mRNA, and protein content. Student-Newman-Keuls post hoc test was used to locate differences. A t-test was applied to evaluate differences in body weight between genotypes within each sex in the single AICAR and single exercise experiment. Before statistical analysis, mRNA data were log transformed to ensure homogeneity of variances. Differences are considered significant at \( P < 0.05 \). Statistical calculations were performed using Sigma Stat statistical software (version 2.03; Sigma Stat, Chicago, IL).

RESULTS

Basal Skeletal Muscle Capillarization

Figure 1 shows a capillary staining of white muscle EDL of WT and PGC-1α KO mice. The C:F in EDL was 20% lower (\( P < 0.05 \)), and the FCSA was 25% lower (\( P < 0.05 \)) in PGC-1α KO mice than in WT mice (table 6). Thus the capillary density (cap/mm²) was similar in PGC-1α KO and WT animals. The VEGF protein content in EDL was 70% lower (\( P < 0.05 \)) in PGC-1α KO mice than in WT mice; however, in accordance with the CD data, no genotype differences were observed in the protein content of the endothelial marker protein CD31 (Table 6).

Exercise Training

No differences between males and females in skeletal muscle VEGF mRNA expression or VEGF, CD31, or HKII protein expression were observed, and therefore, male and female samples were analyzed together.

VEGF mRNA expression. Five weeks of exercise training induced in WT quadriceps an ~60% increase (\( P < 0.05 \)) in VEGF mRNA expression relative to control mice. However, in PGC-1α KO mice, no training-induced changes were detected in VEGF mRNA. In untrained mice, no genotype difference was detected in quadriceps VEGF mRNA content, but in
trained mice the VEGF mRNA expression was 50% higher \((P < 0.05)\) in WT mice than in KO mice (data not shown).

**VEGF protein expression.** In WT mice, 5 wk of exercise training increased \((P < 0.05)\) VEGF protein content \(\sim 50–60\%\) in WG and quadriceps (Fig. 2, A and B). However, no changes were observed in VEGF protein content in PGC-1α KO mice in either of the muscles with training, implying that PGC-1α is crucial for an exercise-induced regulation of VEGF expression. The VEGF protein content in WG and quadriceps was \(\sim 60–80\%\) lower \((P < 0.05)\) in PGC-1α KO mice than in WT both before and after the exercise training regime (Fig. 2, A and B). This finding further strengthens a role of PGC-1α in regulating VEGF protein expression in skeletal muscles.

**CD31 protein expression.** Exercise training was associated with an \(\sim 25\%\) increase \((P < 0.05)\) in CD31 protein content in quadriceps of both WT and PGC-1α KO animals (Fig. 2, C and D), indicating that PGC-1α is not required for a training-induced elevation in endothelial marker protein content, and thus CD3 is likely not required either. No significant training-induced changes were observed in WG in either genotype. Furthermore, no genotype differences were observed in CD31 protein content either in WG or in quadriceps (Fig. 2, C and D).

**HKII protein expression.** HKII protein content was used as a marker of training-induced skeletal muscle adaptations not regulated by PGC-1α and thus an example of a protein equally expressed in PGC-1α KO and WT muscles. HKII data from the exercise training study have been published in a previous work (30). In short, exercise training induced similar increases in HKII protein content in the two genotypes, with \(\sim 45\%\) in WG and \(\sim 130\%\) in quadriceps, and the HKII protein content was similar in KO and WT mice in both untrained and trained animals (30).

**Aging and Lifelong Exercise Training**

**VEGF protein expression.** Aging was associated with an \(\sim 50–60\%\) decrease \((P < 0.05)\) in VEGF protein expression in WG and quadriceps in both genotypes compared with young mice (Fig. 3, A and B).

In WT mice, lifelong exercise training prevented this age-associated decline in VEGF protein expression in both of the investigated muscles, and lifelong exercise-trained WT mice had \(\sim 40\%\) higher \((P < 0.05)\) VEGF protein expression than age-matched controls.

However, in PGC-1α KO mice, lifelong exercise did not prevent this age-associated drop in VEGF protein expression. Thus, VEGF protein expression in quadriceps of lifelong-trained PGC-1α KO mice was \(\sim 50\%\) lower \((P < 0.05)\) than in young PGC-1α KO mice (Fig. 3, A and B), and lifelong exercise-trained PGC-1α KO mice had VEGF protein content similar to that of age-matched PGC-1α controls (Fig. 3, A and B). These findings indicate that PGC-1α is required for the training-induced prevention of the age-induced decline in VEGF expression.

**CD31 protein expression.** Aging had no effect on CD31 expression in any of the muscles in either genotype (Fig. 3, C and D). Furthermore, no significant changes with lifelong exercise training were observed in CD31 protein expression in any of the muscles in either genotype (Fig. 3, C and D). However, it may be noted that a nonsignificant increase in CD31 protein was apparent in lifelong exercise-trained aged WT mice, suggesting that PGC-1α may have some impact on training-induced increased endothelial marker protein expression in muscles of aged mice.

**HKII protein expression.** Aging did not change HKII expression in any of the muscles in either genotype (Fig. 4, A and B), and, as expected, no genotype differences were observed in HKII protein content either in WG or in quadriceps. HKII protein expression in quadriceps of lifelong-trained WT and PGC-1α KO mice was \(25\%\) higher \((P < 0.05)\) than both aged-matched and young controls (Fig. 4B). In WG, however, exercise did not induce any significant changes in HKII protein expression in either WT or PGC-1α KO mice (Fig. 4A).

**Single Exercise Bout**

Endurance exercise training consists of repeated single exercise bouts, and to elucidate the underlying mechanism for a VEGF protein upregulation with endurance exercise training, the effects of a single exercise bout on VEGF mRNA and VEGF protein expression as well as on potential signaling molecules regulating VEGF expression through PGC-1α were examined.
VEGF mRNA and protein expression. A single exercise bout induced a nonsignificant, 20% increase in VEGF mRNA in quadriceps muscle at 6 h of recovery only in WT mice (Fig. 5). No changes were detected in WG VEGF mRNA content at the investigated time points after exercise in either genotype. However, in soleus muscle a single exercise bout induced a 50% increase ($P < 0.05$) in VEGF mRNA content in WT mice, but with no change in PGC-1α KO mice (data not shown). These findings suggest that PGC-1α may be required for exercise-induced VEGF mRNA expression. A single exercise bout did not affect VEGF protein expression in quadriceps muscle at 2 or 6 h of recovery in either genotype (data not shown). This finding indicates that a single exercise bout is not sufficient to elicit detectable changes at the protein level within 6 h of recovery.

AMPK Thr172 and p38 MAPK Thr180/Tyr182 phosphorylation. A single exercise bout induced an 80% increase ($P < 0.05$) in phospho (p)-AMPK Thr172 in WT mice and a 300% increase ($P < 0.05$) in PGC-1α KO mice (Fig. 6A). No genotype difference ($P < 0.05$) in p-AMPK Thr172 was observed before exercise, but p-AMPK Thr172 was ~100% higher ($P < 0.05$) in PGC-1α KO than in WT animals after exercise (Fig. 6A). A single exercise bout increased ($P < 0.05$) p-p38 MAPK Thr180/Tyr182 ~100% in WT mice and PGC-1α KO mice. No genotype difference was observed in p-p38 MAPK Thr180/Tyr182 either before or after exercise (Fig. 6B).

Thus, exercise induces both AMPK Thr172 and p38 MAPK Thr180/Tyr182 phosphorylation in both genotypes. To be able to focus on just the potential role of AMPK Thr172 phosphorylation in PGC-1α-mediated regulation of VEGF expression, an additional experiment with AICAR treatment was conducted.

Single AICAR Treatment

AMPK Thr172 and p38 MAPK Thr180/Tyr182 phosphorylation. Thirty minutes after a single injection of AICAR, p-AMPK Thr172 increased ($P < 0.05$) 60% in WT mice and 160% in PGC-1α KO mice, leading to a 100% higher ($P < 0.05$) p-AMPK Thr172 in PGC-1α KO animals than in WT animals. No genotype difference was observed in p-p38 MAPK Thr180/Tyr182 in either genotype (Fig. 7B). Thus, as expected, the AICAR treatment induced p-AMPK Thr172 and not p-p38 MAPK Thr180/Tyr182.

VEGF mRNA expression. Four hours after a single injection of AICAR, quadriceps VEGF mRNA expression increased ($P < 0.05$) ~100% in WT mice, but without significant change in PGC-1α KO mice (Fig. 8). No genotype difference was observed 4 h after a single AICAR/saline injection. However, these findings support a role of PGC-1α in AICAR-mediated regulation of VEGF mRNA expression.

Repeated AICAR Treatment

VEGF mRNA expression. Repeated AICAR/saline treatment did not induce a significant change in quadriceps VEGF mRNA expression in either genotype. VEGF mRNA expression was ~60% higher ($P < 0.05$) in WT mice after both repeated saline and AICAR treatments (data not shown). These results reveal no detectable cumulative effect on VEGF mRNA in either genotype after repeated AICAR treatment.

VEGF protein expression. As in the other experimental parts, the VEGF protein content in muscles of PGC-1α KO...
mice was lower \((P < 0.05)\) than in WT mice (Fig. 9, A and B). Repeated AICAR treatment in WT mice increased \(P < 0.05\) VEGF protein expression \(\sim 20\%\) in quadriceps (Fig. 9B) and induced a nonsignificant increase in WG VEGF protein expression (Fig. 9A) relative to saline treatment. Because no changes were observed in VEGF protein expression after AICAR treatment relative to saline in PGC-1\(\alpha\) KO-mice, these findings suggest an AICAR/PGC-1\(\alpha\)-mediated regulation of VEGF expression in quadriceps. Together, the VEGF mRNA and protein adaptations to single and repeated AICAR treatments indicate that PGC-1\(\alpha\) is required for the transient VEGF mRNA induction to each treatment, leading to cumulative effects at the protein level, whereas the acute VEGF mRNA responses to AICAR treatment do not lead to an accumulation of VEGF mRNA in the present study.

**CD31 protein expression.** In accordance with the results in the other experimental parts, no genotype-specific difference was observed in CD31 protein expression in either muscle (Fig. 9, C and D). Repeated AICAR treatments had no effect on CD31 protein expression either in WT or in PGC-1\(\alpha\) KO animals, implying that the protocol with repeated AMPK activation was not a sufficient stimulus to increase the expression of this endothelial marker protein.

**HKII protein expression.** No genotype-specific difference was observed in HKII protein expression in either muscle (Fig. 10, A and B). Repeated AICAR treatment of WT and PGC-1\(\alpha\) mice increased \(P < 0.05\) HKII protein expression \(\sim 55\%\) in WG and \(\sim 110\%\) in quadriceps (Fig. 10, A and B). These data show that AICAR induced similar HKII protein adaptations in PGC-1\(\alpha\) KO and WT mice.

**DISCUSSION**

The main findings of the present study are that skeletal muscles of PGC-1\(\alpha\) KO mice have a reduced expression of VEGF protein and that PGC-1\(\alpha\) is a regulatory component in exercise-induced increases in skeletal muscle VEGF expression in both young and aged mice. Furthermore, the results suggest that AICAR-induced regulation of skeletal muscle VEGF expression is mediated by AMPK/PGC-1\(\alpha\) signaling.

**PGC-1\(\alpha\) is Required for Exercise-Induced Increases in VEGF Expression**

Previous studies using cultured muscle cells (58) and transgenic mice (32) have demonstrated that PGC-1\(\alpha\) plays a key role in regulating mitochondrial biogenesis, and a recent study provides evidence that PGC-1\(\alpha\) also affects angiogenesis after an ischemic insult through regulation of VEGF expression (5). Thus, it is possible that PGC-1\(\alpha\) is involved in regulating both exercise-induced mitochondrial biogenesis and angiogenesis in skeletal muscle, thereby coordinating the adaptations in muscle capillarization and oxidative capacity with training (19, 22, 45). In line with this suggestion are the present findings that an exercise-induced upregulation of VEGF mRNA in response to a single exercise bout is PGC-1\(\alpha\) dependent and that repeated exercise bouts (training) increase skeletal muscle VEGF protein expression in WT mice but not in PGC-1\(\alpha\) KO mice. Such a role of PGC-1\(\alpha\) in training-induced VEGF expression is further emphasized by the current finding that PGC-1\(\alpha\) is required for the training-induced prevention of a reduced basal VEGF protein expression in mouse skeletal muscle with age. Thus, lifelong training could prevent the age-associated decline in VEGF expression (31) in skeletal muscle of WT but not PGC-1\(\alpha\) KO mice in the present study. Together, these observations are in agreement with results from experiments on muscle cells in culture showing that PGC-1\(\alpha\) is required for the
induction of VEGF mRNA expression in response to both nutrient and oxygen deprivation (5). Recent work from our laboratory demonstrated that PGC-1α/H9251 is redundant in training-induced increases in expression of several mitochondrial proteins (30). Thus, the present observation that PGC-1α/H9251 is obligatory for the exercise-induced increase in VEGF expression underlines the fact that PGC-1α has a particularly prominent role in regulating training-induced VEGF expression, with no apparent compensatory mechanisms that can take over when PGC-1α is absent.

The present observation that PGC-1α seemed to be required for the mRNA induction of VEGF mRNA after a single exercise bout indicates that PGC-1α mediates exercise-induced transient increases in VEGF mRNA content, leading to an accumulation of VEGF mRNA and protein with training. However, whereas lack of PGC-1α was associated with lowered basal VEGF protein, the basal VEGF mRNA expression was generally similar in PGC-1α KO and WT mice. Thus, PGC-1α does not seem to be necessary for maintaining basal VEGF mRNA levels, and therefore, PGC-1α may also affect VEGF protein expression by mechanisms other than transcriptional regulation of VEGF either directly or indirectly. A possibility for such regulation may be an insulin-mediated effect, because VEGF expression has been shown to be increased by insulin (25), and PGC-1α whole body KO mice have reduced serum insulin levels in the fed state (33).

**PGC-1α is Required for AMPK-Mediated Increases in VEGF Expression**

Exercise provides a large stimulus, and multiple signaling pathways are most likely involved in regulating exercise-induced VEGF expression. Thus, AMPK phosphorylation increased in both genotypes in response to the acute exercise bout, in accordance with previous studies (54). PGC-1α KO mice exhibited a larger exercise-induced increase in AMPK phosphorylation. This finding may be related to the fact that PGC-1α KO mice have lower exercise capacity and thus, are more metabolically stressed by a single exercise bout. Furthermore, this finding may be related to the lower muscle glycogen content previously shown in PGC-1α KO mice at rest and after a single bout of exercise (30), because exercise-induced...
AMPK phosphorylation is enhanced when muscle glycogen is reduced (57). AMPK activation has been shown to direct proangiogenic effects in endothelial cells (36, 37, 46). However, it was recently reported that inactivation of AMPK did not alter the induction of VEGF protein expression or the angiogenic response to exercise (59), implying that additional exercise-induced intracellular signaling pathways other than AMPK-mediated activation of PGC-1α regulate VEGF expression in response to exercise. One such pathway could be an exercise-induced activation of p38 MAPK (15) that has been demonstrated to powerfully phosphorylate and activate PGC-1α (1) and participate in the stabilization of VEGF mRNA (39, 56). Thus, because p38 MAPK is activated by exercise in the present study, it is possible that a p38 MAPK activation of PGC-1α has contributed to the training-induced increase in VEGF protein expression in WT. Furthermore, high-intensity exercise has been shown to increase HIF-1α content and activity (3, 13, 50), and because HIF-1α is a key transcriptional regulator of the VEGF gene, this represents another signaling pathway to increase VEGF expression by exercise training.

To focus on the potential role of an AMPK/PGC-1α-mediated VEGF regulation, an AICAR treatment experiment was conducted. Because a single AICAR injection does not induce HIF-1α in normoxic conditions (29, 38) and a single AICAR injection induced AMPK phosphorylation and not p38 MAPK in the present study (in accordance with previous findings) (1, 20, 54), the AICAR injection protocol applies a model more specific than an exercise protocol to investigate the potential role of AMPK/PGC-1α signaling in regulation of VEGF expression. The present findings that acute activation of AMPK by AICAR increases VEGF mRNA expression and that repeated activation of AMPK by daily injections with AICAR leads to increased VEGF protein expression in mouse skeletal muscle are in agreement with a previous study (38) and suggest a role of AMPK in regulating VEGF expression. In accordance, repeated AICAR injections have been found to upregulate VEGF protein expression in WT but not AMPKα2 KO mice (Jørgensen SB, Richter EA, and Wojtaszewski JFP, unpublished observations). A novel observation in the current study is that the AICAR-induced VEGF expression was abolished in the PGC-1α KO mice, which suggests that AICAR-mediated increases in VEGF expression are in part through AMPK/PGC-1α signaling. Together, these findings put forward that exercise-induced regulation of VEGF expression may involve an AMPK/PGC-1α-dependent signaling pathway, but additional pathways also seem to play a role.

**Basal Capillarization**

Previous studies have suggested that VEGF protein expression is correlated with capillary-to-fiber ratio (59). Furthermore, inactivation of the VEGF gene has been shown to abolish two-thirds of basal skeletal muscle capillarization, measured as capillary-to-fiber ratio and capillary density (49). In accordance, the VEGF protein level that was lower in the PGC-1α KO mice than in WT mice in the present study was associated with a 20% lower capillary-to-fiber ratio, providing evidence for a functional significance of the demonstrated PGC-1α-mediated regulation of VEGF expression. The finding of similar capillary density and protein content of the endothelial cell marker CD31 in PGC-1α KO and WT mice is in accordance with the observed smaller fiber size in the PGC-1α KO mice.

**Capillarization and Exercise Training**

We acknowledge that, to fully understand the vascular adaptations to different interventions, a description of FCSA, capillary density, and capillary-to-fiber ratio is necessary. However, in the exercise training studies, muscle samples for histochemical analysis were not obtained. Instead, as an indication of vascularization, the expression of the endothelial marker CD31 was determined. The present finding that CD31 protein expression was augmented after exercise training in quadriceps of young WT mice is in accordance with previous studies in rodents (2, 7, 35, 47, 53) that show increased capillary density after training. CD31 protein expression was also increased after exercise training in the quadriceps muscle of young PGC-1α KO mice despite the lack of an increase in VEGF protein expression. In accordance, a previous study...
showing that VEGF receptor antagonist treatment decreases, but does not eliminate, training-induced muscle capillarization in rats (34) indicates that capillarization is not simply a function of VEGF availability. Thus, this may explain such apparent dissociation between increases in CD31 and VEGF protein expression in PGC-1α KO mice.

Capillarization and Age

The age-associated loss of muscle capillaries present in humans does not appear to occur in rodents, and the present finding of no changes in CD31 protein with aging in mouse skeletal muscle is in line with previous studies showing no difference in capillary density in old relative to young mice (14, 51).

Previous studies have demonstrated an age-dependent impairment of angiogenesis in response to ischemia (43, 44). However, high-intensity exercise training of old rats has been shown to restore the ability to form new capillaries in response to hypoxia (31). Because exercise training increased VEGF and induced an apparent increase in CD31 protein content in the old WT mice but not in old mice lacking PGC-1α, it is speculated that PGC-1α may play a role in exercise-induced capillary growth, where one potential pathway involves VEGF signaling.

In conclusion, lack of PGC-1α in skeletal muscle is associated with a reduced basal level of VEGF protein as well as a lower capillary-to-fiber ratio in skeletal muscle. Furthermore, PGC-1α is obligatory for exercise-induced upregulation of VEGF expression in skeletal muscle of young mice and for the training-induced prevention of an age-associated decline in VEGF protein expression, emphasizing that PGC-1α plays a major role in regulating exercise-induced VEGF expression. In addition, AMPK activation by AICAR enhanced the expression of VEGF in WT mice only, which could indicate that the exercise-induced increase in VEGF expression may depend on both AMPK and PGC-1α.

ACKNOWLEDGMENTS

We acknowledge the skillful technical assistance of Karina Olsen. We thank Prof. B. Spiegelman for kindly providing PGC-1α+/− mice initially to start breeding in house.

GRANTS

The study was supported by the Lundbeck Foundation, the Novo Nordisk Foundation, the Danish Medical Research Council, and a grant from European Commission FP6 Integrated Project Exgenesis (Ref. no. LSHM-CT-2004-005272). The Centre of Inflammation and Metabolism is supported by the Danish National Research Foundation (Grant no. 02-512-555), The Copenhagen Muscle Research Centre is supported by grants from The University of Copenhagen and Rigshospitalet.

REFERENCES

25. Jorgensen SB, Treebak JT, Violett B, Schjerling P, Vaalont S, Wojtaszewski JF, Richter EA. Role of AMPKα2 in basal, training-, and