Synergistic effect of VEGF gene inactivation in endothelial cells and skeletal myofibers on muscle enzyme activity, capillary supply and endurance exercise in mice

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NEW FINDINGS

1. What is the central question of this study? Dose VEGF expressed by both endothelial cells and skeletal myofibers maintain the number of skeletal muscle capillaries and regulate endurance exercise.

2. What is the main finding and its importance? VEGF expressed by both endothelial cells and skeletal myofibers is not essential for maintaining capillary number but does contribute to exercise performance.

Abstract

Many chronic diseases lead to exercise intolerance, with loss of skeletal muscle capillaries. While many muscle cell types (myofibers, satellite cells, endothelial cells, macrophages and fibroblasts) express VEGF, most muscle VEGF is stored in myofibers vesicles which can release VEGF to signal VEGF receptor-expressing cells. VEGF gene ablation in myofibers or endothelial cells alone does not cause capillary regression. We hypothesized that simultaneously deleting endothelial cell (EC) and skeletal myofiber (Skm) VEGF would cause capillary regression and impair exercise performance. This was tested in adult mice by simultaneous conditional deletion of the VEGF gene (Skm/EC-VEGF-/- mice) through the use of VEGF*LoxP*, HSA-Cre-ER^{T2} and PDGFb-iCre-ER^{T2} transgenes. These double-deletion mice were compared to three control groups – WT, EC VEGF deletion alone and myofiber VEGF deletion alone. Three weeks after initiating gene deletion, Skm/EC-VEGF-/- mice, but not SkmVEGF-/- or EC-VEGF-/- mice, reached exhaustion 40 minutes sooner than WT mice in treadmill tests ($p = 0.002$). WT, SkmVEGF-/-, and EC-VEGF-/-, but not Skm/EC-VEGF-/- mice, gained weight over the three weeks. Capillary density, fiber area and capillary:fiber ratio in soleus, plantaris, gastrocnemius and cardiac papillary muscle were similar across the groups. Phosphofructokinase and pyruvate dehydrogenase activities increased only in Skm/EC-VEGF-/- mice. These data suggest that deletion of VEGF signaling simultaneously in endothelial cells and myofibers, while reducing treadmill endurance and despite compensatory augmentation of glycolysis, is not required for muscle capillary maintenance. Reduced endurance remains unexplained, but may possibly be related to a role for VEGF in controlling perfusion of contracting muscle.

INTRODUCTION

Chronic diseases, such as heart failure, diabetes, chronic obstructive pulmonary disease (COPD) and peripheral arterial disease (PAD) are typically associated with muscle weakness and exercise intolerance (Kao *et al.*, 1994; Jobin *et al.*, 1998; Duscha *et al.*, 1999; Jones *et al.*, 2012; Kato *et al.*, 2015; Aiken *et al.*, 2018). In such conditions, muscle dysfunction can in part be attributed to poor vascular structure and/or function in the peripheral locomotor muscles. For instance, a loss of skeletal muscle capillaries occurs in some patients with severe COPD (Jobin *et al.*, 1998; Jatta *et al.*, 2009). Similarly in chronic heart failure patients, it has been proposed that, in addition to central limitations in the O_2 transport system, microvascular function is compromised by fewer capillaries supporting red blood cell perfusion and a lower capillary hematocrit that reduce O_2 availability and utilization by muscle mitochondria (Copp *et al.*, 2012; Hirai *et al.*, 2015). In peripheral artery disease, muscle function may depend not only on perfusion through functioning capillaries, but the formation of collateral arteries to circumvent occluded vessels (Takeda *et al.*, 2011; Kofler & Simons, 2015). In all these chronic conditions, inhibited VEGF signaling has been implicated in regulating vascular function and structure (Gustafsson *et al.*, 2001; Jatta *et al.*, 2009; Esposito *et al.*, 2010; Jones *et al.*, 2012; Kikuchi *et al.*, 2014).

However, the mechanism leading to skeletal muscle capillary regression due to detraining or chronic disease conditions is not well understood. Skeletal muscle is unique in that the dynamic regulation of capillaries is a normal physiologic response to exercise training, and VEGF expressed by mature skeletal myofibers is essential for both the angiogenic and metabolic changes that occur with daily running exercise (Delavar *et al.*, 2014). Skeletal myofibers produce large quantities of VEGF that is released by exercise in extracellular vesicles to potentially signal myofiber associated endothelial cells, macrophage and satellite cells in peripheral muscle and as a myokine that effects both cardiac and cerebral function (Hoier *et al.*, 2013; Delavar *et al.*, 2014; Knapp *et al.*, 2016; Tang *et al.*, 2016;

Rich *et al.*, 2017; Nie *et al.*, 2019). Interestingly, VEGF gene deletion in adult mice, targeted to skeletal myofibers, decreases VEGF content up to 90%, depending on the skeletal muscle type , but, no capillary regression occurs after eleven weeks (Delavar *et al.*, 2014). Loss of efficient muscle contraction-induced perfusion of the muscle suggests that microvascular function is compromised (Knapp *et al.*, 2016). In contrast, earlier studies from our laboratory, using a viral delivery system to express *cre* recombinase in the gastrocnemius of adult VEGF*LoxP* mice, suggested that inhibition of VEGF expression can initiate capillary regression. In this previous study, the viral vector was capable of infecting any cell type located within the muscle (Tang *et al.*, 2004). In addition mice exposed to cigarette smoke, the main risk factor for COPD, exhibit both loss of capillaries and lower VEGF levels in the oxidative soleus muscle but not the more glycolytic, EDL (Tang *et al.*, 2010b). Whether paracrine signaling from supporting myofibers acts to protect vascular endothelial cells, and/or functions in concert with autocrine VEGF signaling to prevent the loss of capillary structures in skeletal muscle has not been tested in an adult *in vivo* model.

Thus, in this study we hypothesized that the simultaneous inhibition of VEGF expression in endothelial cells and skeletal myofibers in adult mice would lead to capillary regression and limit exercise capacity. This hypothesis was tested by conditionally deleting the VEGF gene with a tamoxifen-inducible Cre-LoxP system in: 1. endothelial cells alone, 2. skeletal myofibers alone, and 3. simultaneously in both these cell types. Exercise capacity was evaluated by treadmill tests of maximum speed and endurance performance before and after inducing VEGF gene deletion. Vascular and myofiber alterations that could contribute to exercise limitation were measured. These included an analysis of the capillary density, fiber size and type and the capillary to fiber ratio in several locomotor muscles and the heart and skeletal muscle metabolic enzyme activities.

MATERIALS AND METHODS

Ethical Approval

This study was reviewed and approved by the University of California, San Diego, Animal Care and Use Committee, Protocol S01144. VEGF*LoxP* mice, in which loxP sites are located in exon 3 of the VEGF-A gene, were a generous gift from Genentech (Dr. Ferrara) (Gerber *et al.*, 1999). HSA-Cre-ER^{T2} mice were a generous gift from Dr. Chambon's laboratory (Schuler *et al.*, 2005). PDGFb-iCRE-ER^{T2} mice on a C57Bl x CB6 background were from Dr. Fruttiger's laboratory (Claxton *et al.*, 2008). All mouse lines were backcrossed onto a C57BL/6J background. Mice were housed four to five per cage in the same room in a pathogen-free vivarium with a 12:12 h day-night cycle and were provided with standard mouse chow (Harlan Tekland 8604, Madison, WI) and water *ad libitum*.

The inducible endothelial cell specific VEGF deficient (EC-VEGF -/-) mouse model was developed by crossbreeding two separate transgenic lines: homozygous VEGF*LoxP+/+* and heterozygous PDGFb-iCRE-ER^{T2}+/- mice. The inducible myofiber specific VEGF deficient (SkmVEGF-/-) mice were created through crossbreeding VEGF*LoxP+/+* and heterozygous HSA-Cre-ER^{T2}+/- mice as previously described (Delavar *et al.*, 2014). The inducible combined endothelial and myofiber VEGF deficient (Skm/EC-VEGF-/-) animals were obtained by the crossbreeding of mice that were heterozygous for both the PDGFb-iCRE-ER^{T2} or HSA-Cre-ER^{T2} transgenes and all on a homozygous VEGF*LoxP* background. In PDGFb-iCRE-ER^{T2}+/- mice, the GFP reporter linked to the PDGFb-iCRE-ER^{T2} transgene was detected in 91% and 85% of the CD31+ endothelial cells located in the soleus and gastrocnemius, respectively. VEGF*LoxP* mice without a *cre* recombinase gene sequence were used as the littermate, control group.

Genotyping. DNA was extracted from mouse tail-sections (DNeasy Tissue Kit, Qiagen Inc., Valencia, CA, USA) and used for PCR analysis to identify mouse genotypes. The following primer sequences were used: VEGF*LoxP*, forward primer 5'-TCCGTACGACGCATTTCTAG-3' and reverse primer 5'-

CCTGGCCCTCAAGTACACCTT-3'; for HSA-Cre-ER^{T2} transgene, forward primer 5'-CTAGAGCCTGTTTTGCACGTTC-3' and reverse primer 5'-TGCAA-GTTGAATAACCGGAAA-3'; for PDGFb-iCRE-ER^{T2} recombinase, forward primer 5'-CCAGCCGCCGTCGCAACTC-3' and reverse primer 5'-GCCGCCGGGATCACTCTCG-3'. The following PCR conditions were used for VEGF*LoxP* and HSA-Cre-ER^{T2} transgenes: a 2-minute polymerase activation incubation at 95° C, then 36 cycles of 30 s at 94° C for denaturation, 30 s of annealing at 52° C, 60 s at 72° C for elongation, concluding with 10 min. at 72° C. The PCR conditions used for PDGFb-iCre-ER^{T2} transgene were the same as above differing only in the annealing temperature, 59° C. All PCR products were then electrophoresed on a 2% agarose gel stained with ethidium bromide (NuSieve GTG/SeaKem HGT agarose, Cambrex Bio Science Rockland, Inc., Rockland, ME, USA) in 1X Tris-Acetate-EDTA (TAE) buffer.

Experimental Design. Four experimental mouse groups were studied, defined by different genotypes as follows: skeletal myofiber specific VEGF gene deletion (SkmVEGF-/-), endothelial cell specific VEGF gene deletion (EC-VEGF-/-), combined endothelial and skeletal myofiber (Skm/EC-VEGF-/-), and control littermates (WT). At 4 months of age, male mice were weighed, exercise tested and then treated with tamoxifen (1 mg/mouse, i.p) for five days (Day 0 - 4) to ablate the VEGF gene. WT mice, which did not carry a *cre* recombinase gene, were also treated with tamoxifen to control for any effects of this agent. Twenty-one days after initiating tamoxifen treatment to allow sufficient time for Cre recombinase mediated gene deletion between LoxP sites (Schuler *et al.*, 2005), mice were weighed and exercise tested. Mice were then anesthetized with 2.5% isoflurane and hind limb skeletal muscles [soleus, plantaris, gastrocnemius, extensor digitorum longus (EDL)] and heart were removed, weighed, frozen in liquid nitrogen, and stored at −80°C for later analysis or preparation of cryosections.

Aerobic Exercise Performance. Mice were familiarized with the treadmill (Model CL-4, Omnitech, Columbus, OH) twenty-four hours before exercise performance tests. The familiarization consisted

of allowing mice to run for 10 minutes at 10 cm/s on a 10° incline. Exercise performance was evaluated through two different tests: a maximal speed test and an endurance test. These tests were performed on consecutive days. Maximal speed was determined by running mice on a treadmill at 33 cm/min on a 10° incline for 1 minute, and then increasing the speed by 3-4 cm/s each minute until exhaustion. Endurance capacity was measured as the time to reach exhaustion when mice were run at 36 cm/s (60% of the average maximal speed for the control group). It should be noted interbreeding these various Cre driver lines resulted in a WT group with greater endurance (reaching similar endurance times at a greater relative intensity) than our previous studies with VEGF*LoxP* X HAS-Cer-ER^{T2} mice (Delavar *et al.*, 2014; Knapp *et al.*, 2016). The criterion for exhaustion was the inability of the mouse to maintain its position on the treadmill for 8 consecutive seconds.

Muscle Morphology. Skeletal muscles (gastrocnemius, soleus and plantaris) and the heart were surgically removed from mice 3 weeks after initiating tamoxifen administration. Cryosections (10 µm) were prepared from cross-sections of the peripheral muscles. Heart cross-sections were prepared from the upper region of the ventricles and the papillary muscle analyzed. Capillaries and fibers were detected using the Capillary Lead-ATPase method (Rosenblatt *et al.*, 1987). Stained sections were digitally viewed and stored using Hamamatsu Nanozoomer Slide Scanning System. Capillary to fiber ratio, capillary density, fiber-cross sectional area, and fiber-type composition were analyzed from the entire cross-sections using ImageJ software. Gastrocnemius complex 10 µm cryosections were prepared to detect α-smooth muscle actin+ cells (Sigma, St. Louis, MO). Sections were fixed with 4% PFA, washed in tris-buffered saline (TBS) (3x for 5 min.), blocked in 3% BSA in tris-buffered saline (TBS), incubated overnight in mouse anti-actin, α-smooth muscle antibody (1:200) in blocking solution at 4°C. Sections were washed in TBS and then treated with Alexa Fluor 488 secondary detection antibody (1:1000) (Invitrogen, Carlsbad, CA). Sections were then analyzed for α-smooth muscle actin (arterial remodeling), and stained with DAPI (4', 6-diamidino-2-

phenylindole, Invitrogen, Carlsbad, CA) to visualize nuclei. Stained sections were digitally viewed and analyzed using Hamamatsu Nanozoomer Slide Scanning System.

VEGF Protein Levels. VEGF was measured using a mouse specific VEGF ELISA kit (R&D Systems, La Jolla, CA). Preparation of soleus and gastrocnemius extracts was performed as previously described in our laboratory (Tang *et al.*, 2010a). In brief, the lateral gastrocnemius muscle was homogenized in (50 µl/mg) extraction buffer (100 mM of K_2HPO_4, 5 mM of $MgSO_4$, and 30 mM of NaF) (Baldwin *et al.*, 1973) and centrifuged at 10,000 g for 10 minutes at 4° C. The supernatant was used to measure VEGF levels and metabolic enzyme activities (described below). VEGF levels were normalized to total protein levels measured using the DC-Protein Assay (Bio-Rad).

Metabolic Enzyme Analysis. Total enzyme activity levels of phosphofructokinase (PFK) and pyruvate dehydrogenase (PDH) were measured in soluble muscle homogenates (Baldwin *et al.*, 1973; Tang *et al.*, 2010a) of the lateral gastrocnemius. PFK was measured according to the method of Lowry (Lowry *et al.*, 1978). Pyruvate dehydrogenase activity was measured with pyruvate dehydrogenase (PDH) enzyme activity microplate assay kit (Abcam, Cambridge, MA). Enzyme activity was recorded by spectrophotometer (Beckman DU-640) readings at room temperature and expressed in units of catalyzing µmol substrate/ mg tissue/

Statistics. A one-way ANOVA was used to detect differences between the four experimental groups for measurements of VEGF, muscle weight, morphometric parameters, and metabolic enzyme activities. A two-way repeated measures ANOVA was used to detect differences between the four experimental groups treadmill exercise performance both before and after tamoxifen. A Tukey post-hoc test was used to analyze specific differences between the four experimental groups. $P < 0.05$ was considered significant. Data are expressed as the mean +/- SD.

RESULTS

Skeletal muscle VEGF levels in skeletal myofiber-targeted, endothelial-targeted, and combined endothelium and skeletal myofiber-targeted mice.

In order to confirm recombination efficiency, we measured VEGF protein levels in skeletal muscle by ELISA. VEGF levels in the soleus of SkmVEGF-/-, EC-VEGF-/-, and Skm/EC-VEGF-/- mice were 79, 53, and 82%, respectively, lower than VEGF levels in WT soleus. The medial gastrocnemius VEGF levels in SkmVEGF-/- and Skm/EC-VEGF-/- mice were 94 and 97%, respectively, lower than VEGF levels of the WT medial gastrocnemius but unchanged from WT levels in EC-VEGF-/- mice (Figure 1).

Aerobic endurance capacity in Skm/EC-VEGF-/- mice

To assess the functional consequences of VEGF deletion in the different target cells, treadmill exercise performance was measured and before and three weeks after initiating VEGF gene deletion with tamoxifen. Maximal running speed was not different across all four experimental groups of mice (Figure 2A). The was an overall effect on running speed between before initiating gene deletion with tamoxifen and 3 weeks after tamoxifen treatment. In treadmill running endurance tests, the time to reach exhaustion of Skm/EC-VEGF-/- mice three weeks after tamoxifen treatment was 46% (or 40 minutes) lower than tamoxifen treated WT mice (WT, 73.5 ± 6.4 min, Skm/EC-VEGF-/-, 33.5 ± 7.5 min, $p = 0.002$). Whereas, endurance times for SkmVEGF-/- and EC-VEGF-/- mice were not significantly different from WT mice (Figure 2B).

Body mass in mice with combined endothelial cell and skeletal myofiber VEGF gene deletion

WT, SkmVEGF-/-, and EC-VEGF-/- mice gained weight over the three-week period after administration of tamoxifen. In contrast, the body mass of Skm/EC-VEGF-/- mice remained unchanged (Table 1). Skm/EC-VEGF-/- mice had larger heart to body mass ratios than SkmVEGF-/-, EC-VEGF-/-, and WT mice ($p = 0.049$), but absolute heart mass was not different between groups. Skeletal muscle absolute mass and mass to body mass ratio were not altered in the soleus, plantaris, gastrocnemius, and EDL muscles across all groups (Table 1).

Skeletal muscle morphology and fiber type composition

The capillary to fiber ratio, capillary density, and fiber cross-sectional area in skeletal muscle (soleus, plantaris, and gastrocnemius) and the heart (papillary muscle) were not different across the four experimental groups (Table 2). Fiber type composition measured in skeletal muscle types was also not different (Table 3). There were no differences in the number of α-smooth muscle actin positive arteries per skeletal muscle cross sectional area between WT and Skm/EC-VEGF-/- mice. Furthermore, the distribution of arterial sizes was the same in WT and Skm/EC-VEGF-/- mice (Figure 3).

Glycolytic enzyme activities in the gastrocnemius

In the lateral gastrocnemius of Skm/EC-VEGF-/- mice, the activities of phosphofructokinase (PFK) and pyruvate dehydrogenase (PDH) were increased by 46% and 71%, respectively ($P < 0.05$), above the levels measured in muscle homogenates from WT mice. However, the PFK and PDH activities of the lateral gastrocnemius of SkmVEGF-/- and EC-VEGF-/- mice were not different than the WT muscle (Figure 4).

DISCUSSION

The major purpose of this study was to determine whether conditional deletion of the VEGF gene, simultaneously in both endothelial cells and myofibers of adult mice, (Skm/EC-VEGF-/-) would lead to capillary regression and exercise intolerance. The major findings of this study are, in adult mice, that: 1) VEGF deletion in endothelial cells or myofibers alone does not impair running endurance or muscle capillarity. 2) The deletion of VEGF in both cell types simultaneously impairs treadmill endurance, demonstrating synergy between cell types, but does not reduce capillarity. 3) Mice deficient in both endothelial cell and myofiber VEGF exhibit a metabolic response manifested by an up-regulation of glycolytic enzymes and attenuated gain in body mass. However, VEGF deletion, separately, in endothelial cells or myofibers does not produce these consequences. Thus, the principal finding in this study is that *in vivo* VEGF signaling by multiple cells localized in the skeletal muscle is important for regulating metabolism and overall exercise tolerance.

Inhibition of VEGF expression in both endothelial cells and skeletal myofibers does not lead to capillary regression. Contrary to our original hypothesis, inhibition of VEGF expression in endothelial cells and/or skeletal myofibers over a three-week period does not appear to be essential for maintaining capillaries in adult mice. Capillary morphology was assessed in both oxidative and glycolytic muscle types, and in all situations, the capillary to fiber ratio was similar across groups. This result is similar to a previous study in our laboratory in which we reported that skeletal myofiber VEGF was not necessary to maintain capillaries in adult cage-confined mice (Delavar *et al.*, 2014) and the studies of Kamba et al, (Kamba *et al.*, 2006) using AG-013736, a small-molecule VEGFR tyrosine kinase inhibitor or the soluble VEGF receptor for a similar time frame of three weeks. In the VEGF receptor inhibition study skeletal muscle (tongue), heart and brain were found to be

resistant to capillary regression. However, it should be noted that in adult mice skeletal myofiber VEGF is essential for the angiogenic response to 8 weeks of daily exercise training on a treadmill (Delavar *et al.*, 2014), and skeletal myofiber VEGF is necessary for maintaining capillaries in plantaris undergoing hypertrophy in response to an overload stimulus (Huey *et al.*, 2015). Thus, the mechanism leading to capillary regression differs from that initiating skeletal muscle angiogenesis in response to exercise or injury.

Skeletal muscle microvasculature. The number of capillaries did not change following combined skeletal myofiber of endothelial-targeted VEGF gene deletions. Thus unlike capillaries beds that are still developing or highly fenestrated as in turmors or the pancrease, skeletal muscle capillaries are structurally resistant to VEGF inhibition though high resolution of the morphology has yet to be evaluated. (Oki *et al.*, 1999; Kamba *et al.*, 2006; Baum *et al.*, 2017). If there is a high inflammatory burden, such as occurs following cigarette-smoke exposure, capillaries can regress in the oxidative soleus, accompanied by lower VEGF levels (Tang *et al.*, 2010a; Nogueira *et al.*, 2018). Finally, our earlier study using a non-cell specific AAV-Cre directly injected into the gastrocnemius of VEGF*LoxP* mice resulted in an approximately 60% loss of capillaries (Tang *et al.*, 2004). Taken together these data suggest that additional inflammatory or anti-angiogenic factors or possibly VEGF expressed by other cell types, i.e. macrophage, fibroblast, satellite cells, play a role in the maintenance of skeletal muscle capillaries.

The contribution of VEGF expression to endurance exercise. However, irrespective of a change in peripheral muscle capillary number, mice with combined deletion of the VEGF gene in endothelial cells and myofibers reached exhaustion during the endurance tests in about half the time of the control group. Factors that could contribute to a decrease in endurance include central limitations to maximal oxygen uptake; a change in microvascular structure or function that alters the flux of O_2

from microvessels to myofibers (Knapp *et al.*, 2016); and decreased mitochondrial electron transport activity or biogenesis. (Holloszy & Coyle, 1984; Rapoport, 2010; Caffin *et al.*, 2013; Rowe *et al.*, 2013). Exercise training improves endurance in part through a greater utilization of fatty acids as a substrate and this allows the preservation of carbohydrate reserves, glycogen and blood glucose (Love *et al.*, 2011; Lindholm *et al.*, 2014). We have previously reported that VEGF gene deletion targeted to adult skeletal myofibers blunts contraction-induced perfusion of the capillary bed in exercise intolerant mice (Knapp *et al.*, 2016). Down regulation of intracellular VEGF in cultured endothelial cells promotes autophagic cell death and evidence of autophagic vacuoles could be detected by electron microscopy in the endothelial cells of mice with inducible endothelial targeted VEGF gene deletion (Domigan *et al.*, 2015). Thus, VEGF expressed by both endothelial cells and myofibers plays a role in the integrity and function of cardiac and skeletal muscle capillaries, and the impact on exercise performance is greatest with VEGF deficiency in both cell types.

Metabolic consequences of altered VEGF-dependent capillary function

Endothelial cells with knockdown of intracellular VEGF *in vitro* exhibit metabolic changes that included decreased glucose uptake, lactate production, and triglyceride synthesis (Domigan *et al.*, 2015). Basal cellular oxygen consumption and mitochondrial respiratory capacity were also reported to be decreased (Domigan *et al.*, 2015). In the present study when VEGF gene deletion takes place in both endothelial cells and myofibers, PFK and PDH activity increases in glycolytic or mixed locomotor muscles and, this would suggest a preferential oxidation of glucose, from glycogen stores or blood glucose. PDH is a critical enzyme for determining whether acetyl-CoA derived from glucose or fatty acids enters into the TCA cycle (Chambers *et al.*, 2011; Vacanti *et al.*, 2014), and mice with constitutively active PDH have been shown to down regulate beta-oxidation of fatty acids (Rahimi *et al.*, 2014). An increase in both PFK, the rate-limiting enzyme in glycolysis, and PDH would suggest

that glucose transported into the cell, undergoes glycolysis, and is either converted to lactate or to pyruvate and enter the TCA cycle. Pyruvate can then either be transaminated to alanine, undergo an anaplerotic reaction to oxaloacetate or oxidized through the electron transport chain (Gibb *et al.*, 2017). An increase in PDH activity without enhanced mitochondrial respiration of pyruvate or fatty acids could activate ancillary glycolytic mechanisms as occurs in hind-limb ischemia revascularization (Ganta *et al.*, 2017). Further studies are required to uncover these precise metabolic pathways that compensate for inhibited VEGF signaling in sedentary mice but are ultimately in sufficient to avoid impaired endurance.

Summary. These studies highlight the importance of VEGF expressed in both vascular endothelial cells and the skeletal myofiber for regulating key vascular processes that limit exercise capacity. Adaptive changes in skeletal muscle metabolism and vascular function are likely to be main contributing factors to greater exercise intolerance.

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ADDITIONAL INFORMATION

Data Availability: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing Interests: The authors do not have any perceived or potential conflict of interest, financial or otherwise.

Author Contributions: Experimental Design; PDW and ECB. Mouse Model; MF, Data Collection and Analysis; AS, JF, AV-M, ECB, Manuscript preparation and editing; AS, JF, AV-M, PDW, MF, ECB.

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Table 1. Muscle Mass to Body Mass Ratios

		WT	SkmVEGF	EC-VEGF	Skm/EC-VEGF
Heart	mg	155.2 ±0.108	158.1 ± 0.037	143.4 ± 0.022	156.6 ± 0.029
	mg/BM	5.6 ± 0.6	5.46 ± 0.57	5.46 ± 0.56	6.17 ± 0.1.1*****
	n	36	17	31	24
Soleus	mg	11.4 ± 5.6	13.3 ± 5.3	10.2 ± 5.5	13.1 ± 8.4
	mg/BM	0.42 ± 0.23	0.63 ± 0.50	0.39 ± 0.25	0.54 ± 0.45
	n	32	15	24	21
Plantaris	mg	23.8 ± 6.9	23.4 ± 7.7	20.3 ± 9.1	20.0 ± 6.0
	mg/BM	0.88 ± 0.28	1.09 ± 0.70	0.78 ± 0.34	0.80 ± 0.18
	n	32	15	24	21
Gastrocnemius	mg	128.0 ± 19	127 ± 21	131 ± 31	121 ± 22
	mg/BM	4.66 ± 0.67	5.25 ± 0.81	4.95 ± 1.09	4.75 ± 1.24
	n	32	15	27	23
EDL	mg	12.4 ± 7.5	12.4 ± 2.9	15.8 ± 13	9.51 ± 2.6
	mg/BM	0.45 ± 0.34	0.52 ± 0.19	0.61 ± 0.51	0.36 ± 0.09
	n	32	15	27	21
BM	Pre-Tam	26.1 ± 1.9	26.6 ± 2.4	25.3 ± 2.1	25.2 ± 2.2
	Post-Tam	27.6 ± 2.1#	28.4 ± 2.6#	26.7 ± 2.4#	25.5 ± 3.1
	n	36	24	31	17

Values are mean ± SD. * indicates a significant difference ($p = 0.049$). # indicates a difference between Pre-Tam and Post-Tam ($p<0.001$)

Table 2. Skeletal and Cardiac Muscle Morphology

		WT	SkmVEGF	EC-VEGF	Skm/EC-VEGF
Capillary/Fiber Ratio	Soleus	1.86 ± 0.30	1.81 ± 0.24	1.73 ± 0.24	1.68 ± 0.33
	Plantaris	1.24 ± 0.30	1.41 ± 0.12	1.31 ± 0.24	1.28 ± 0.3-
	Gastrocnemius	1.22 ± 0.33	1.42 ± 0.15	1.27 ± 0.13	1.28 ± 0.21
	Papillary	2.00 ± 0.07	1.97 ± 0.02	1.95 ± 0.05	1.99 ± 0.03
Mean Fiber Area (μm^2) x 1000	Soleus	1.88 ± 0.40	2.00 ± 0.56	1.81 ± 0.26	1.84 ± 0.69
	Plantaris	2.26 ± 0.63	2.22 ± 0.40	2.08 ± 0.37	1.97 ± 0.30
	Gastrocnemius	3.66 ± 1.5	3.07 ± 1.6	3.27 ± 0.42	2.94 ± 0.69
	Papillary	5.97 ± 1.5	5.84 ± 1.1	5.51 ± .95	6.64 ± 0.63
Capillary Density (cap/mm^2)	Soleus	962 ± 292	888 ± 284	950 ± 230	863 ± 276
	Plantaris	578 ± 259	655 ± 140	663 ± 209	659 ± 174
	Gastrocnemius	362 ± 179	437 ± 142	400 ± 79	459 ± 144
	Papillary	348 ± 70	354 ± 56	363 ± 56	301 ± 276

Values are mean ± SD. (n = 6-11). No significant differences in any of the three indices for any muscle across the four groups

Table 3. Skeletal Muscle Fiber Type Composition

		Fiber Type Ratio		
		I	**IIa**	**IIb**
Soleus	WT	0.49 ± 0.02	0.49 ± 0.02	0.01 ± 0.01
	SkmVEGF-/-	0.50 ± 0.03	0.49 ± 0.02	0.01 ± 0.01
	EC-VEGF-/-	0.44 ± 0.02	0.54 ± 0.02	0.01 ± 0.01
	Skm/EC-VEGF-/-	0.44 ± 0.02	0.54 ± 0.02	0.01 ± 0.01
Plantaris	WT	0.21 ± 0.01	0.27 ± 0.02	0.52 ± 0.03
	SkmVEGF-/-	0.19 ± 0.03	0.28 ± 0.03	0.53 ± 0.03
	EC-VEGF-/-	0.19 ± 0.02	0.23 ± 0.03	0.58 ± 0.03
	Skm/EC-VEGF-/-	0.20 ± 0.04	0.28 ± 0.04	0.48 ± 0.02
Gastrocnemius	WT	0.02 ± 0.01	0.05 ± 0.01	0.92 ± 0.02
	SkmVEGF-/-	0.03 ± 0.01	0.03 ± 0.01	0.95 ± 0.02
	EC-VEGF-/-	0.01 ± 0.01	0.02 ± 0.01	0.97 ± 0.01
	Skm/EC-VEGF-/-	0.03 ± 0.01	0.05 ± 0.01	0.91 ± 0.02

Values are mean ± SEM. (n = 6-12). No significant differences in any of the three indices for any muscle across the four groups

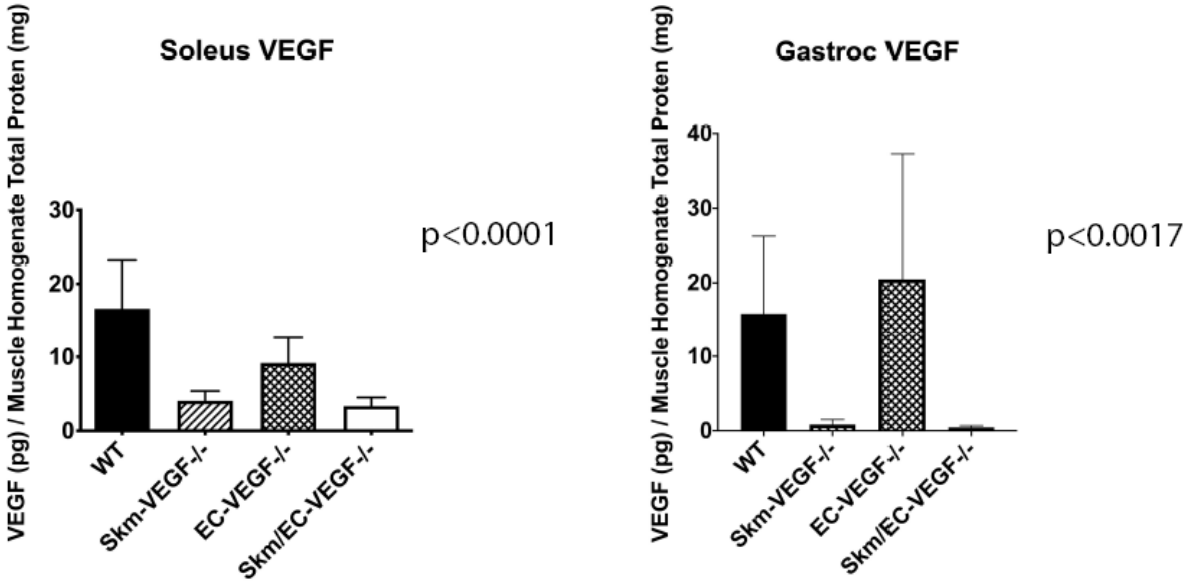

Figure 1. Skeletal muscle VEGF levels. VEGF protein levels in the (A) soleus and (B) medial gastrocnemius of WT, EC-VEGF-/-, SkmVEGF-/-, and Skm/EC-VEGF-/- mice. Values are the mean ± SD. soleus, n=6-18; medial gastrocnemius, n= 3-10.

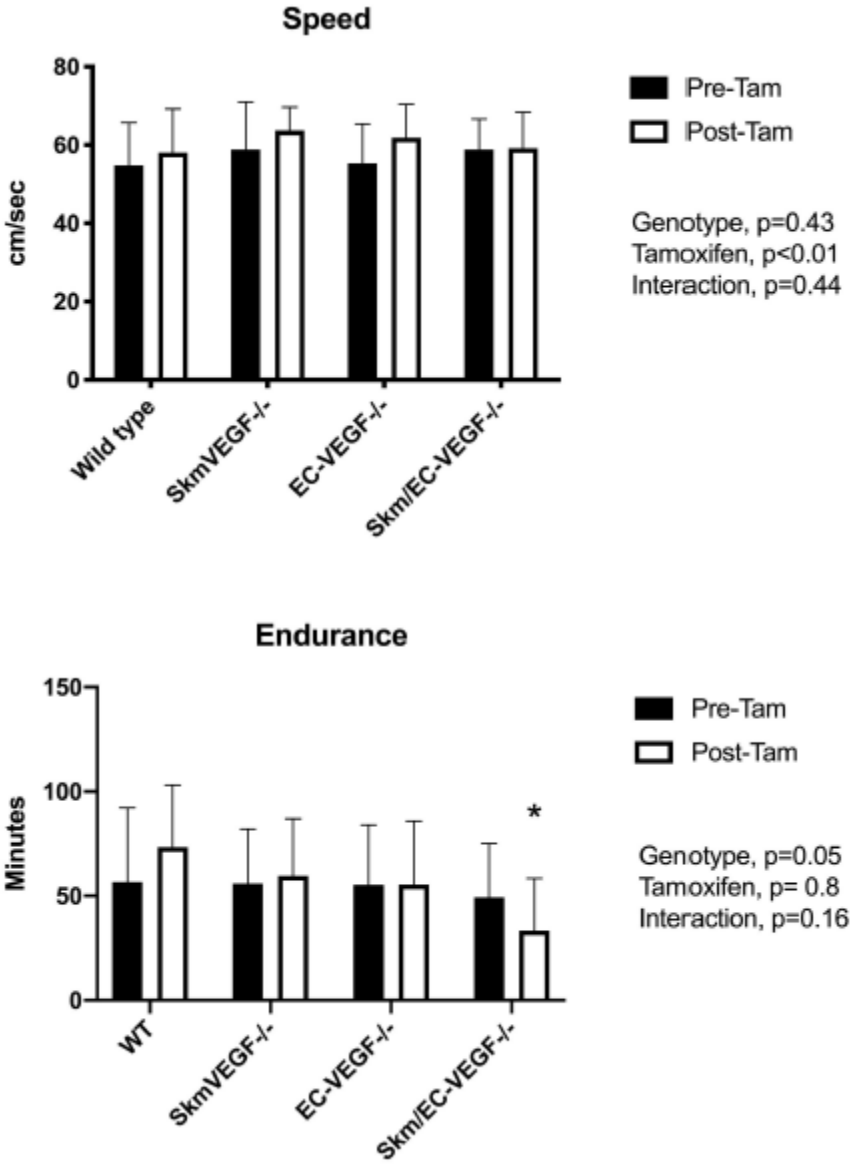

Figure 2. Treadmill exercise performance. WT, SkmVEGF-/-, EC-VEGF-/-, and Skm/EC-VEGF-/- mice were subjected to (A) maximal speed and (B) endurance tests before and three weeks after imitating gene deletion with tamoxifen. Values are mean ± SD. * indicates a significant difference ($p = 0.002$) between WT and Skm/EC-VEGF-/- mice after tamoxifen (n=11-30).

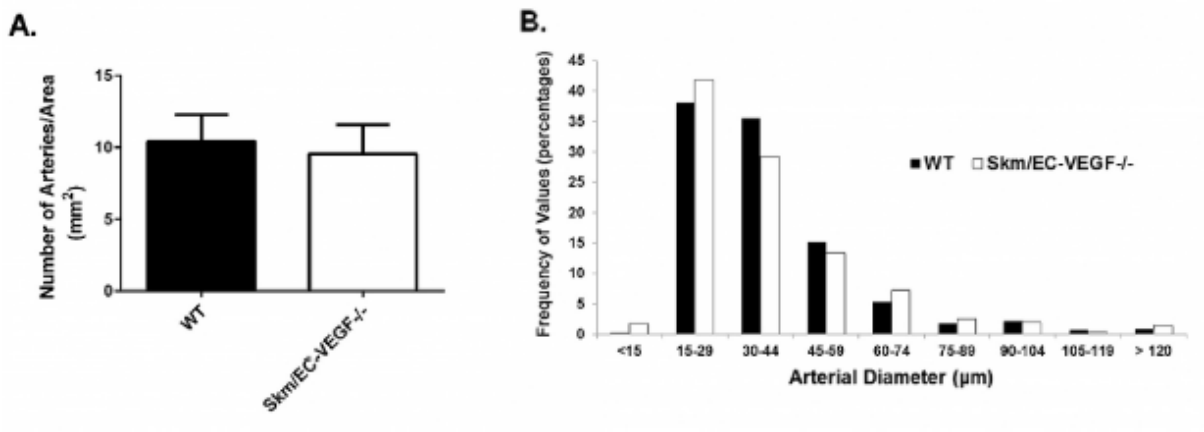

Figure 3. Small Artery Number and Distribution is Maintatined in Skm/EC-VEGF-/- mice. (A) Total α-smooth muscle actin+ vessels per area (mm^2) and (B) histogram of the distribution of arterial diameter sizes (μm) in WT and Skm/EC-VEGF-/- gastrocnemius complexes. Values are the mean $\pm$ SEM (n=5).

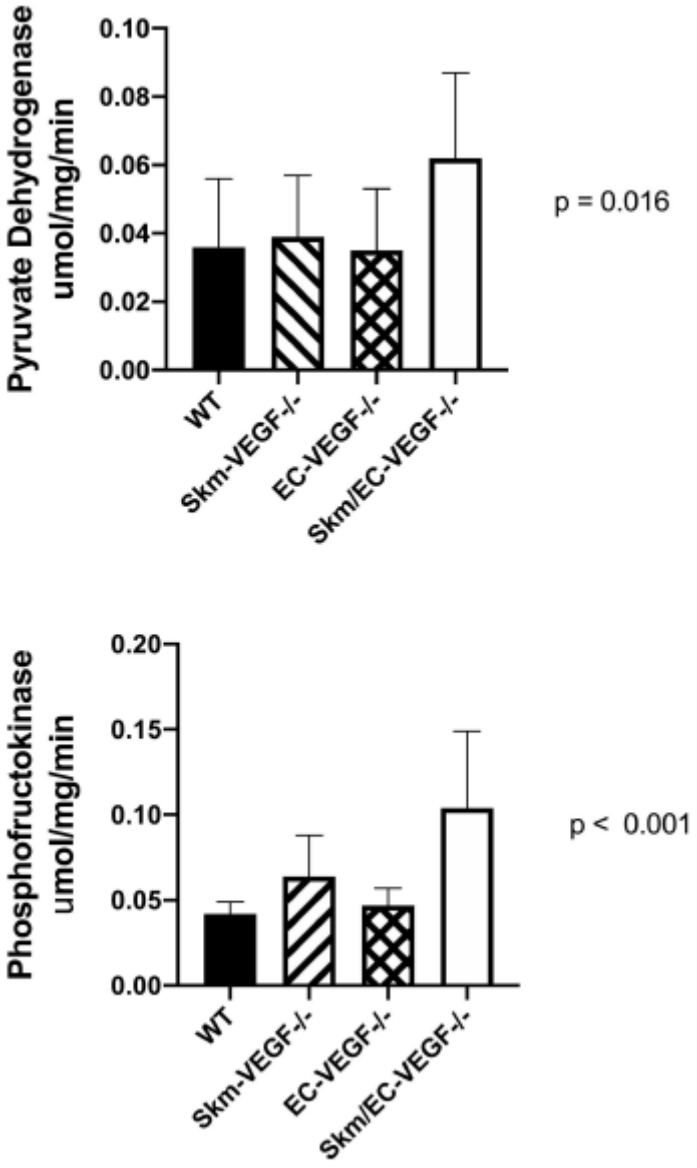

Figure 4. Metabolic Enzyme Activities. (A) Pyruvate dehydrogenase and (B) phosphofructokinase activities (μmol/mg/min) in the lateral gastrocnemius. Values are mean ± SEM. * indicates a significant difference ($p<0.05$) from WT values (n=10).