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Skeletal myofiber VEGF is essential for the exercise training response in adult mice

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Delavar H, Nogueira L, Wagner PD, Hogan MC, Metzger D, Breen EC. Skeletal myofiber VEGF is essential for the exercise training response in adult mice. *Am J Physiol Regul Integr Comp Physiol* 306: R586–R595, 2014. First published February 12, 2014; doi:10.1152/ajpregu.00522.2013.—Vascular endothelial growth factor (VEGF) is exercise responsive, pro-angiogenic, and expressed in several muscle cell types. We hypothesized that in adult mice, VEGF generated within skeletal myofibers (and not other cells within muscle) is necessary for the angiogenic response to exercise training. This was tested in adult conditional, skeletal myofiber-specific VEGF gene-deleted mice (skmVEGF−/−), with VEGF levels reduced by >80%. After 8 wk of daily treadmill training, speed and endurance were unaltered in skmVEGF−/− mice, but increased by 18% and 99% ($P < 0.01$), respectively, in controls trained at identical absolute speed, incline, and duration. In vitro, isolated soleus and extensor digitorum longus contractile function was not impaired in skmVEGF−/− mice. However, training-induced angiogenesis was inhibited in plantaris (wild type, 38%, skmVEGF−/− 18%, $P < 0.01$), and gastrocnemius (wild type, 43%, $P < 0.01$; skmVEGF−/−, 7%, not significant). Capillarity was maintained (different from VEGF gene deletion targeted to multiple cell types) in untrained skmVEGF−/− mice. Arteriogenesis (smooth muscle actin+, artery number, and diameter) and remodeling [vimentin+, 5′-bromodeoxycytidine (BrdU)+, and F4/80+ cells] occurred in skmVEGF−/− mice, even in the absence of training. skmVEGF−/− mice also displayed a limited oxidative enzyme [citrate synthase and β-hydroxyacyl CoA dehydrogenase (β-HAD)] training response; β-HAD activity levels were elevated in the untrained state. These data suggest that myofiber expressed VEGF is necessary for training responses in capillarity and oxidative capacity and for improved running speed and endurance.

angiogenesis; exercise; metabolism; peripheral vascular disease

EXERCISE INTOLERANCE is exhibited by most patients with peripheral arterial disease, chronic obstructive pulmonary disease, and diabetes and may stem from poor peripheral circulation. In these chronic conditions, reduced oxygen and nutrient availability to skeletal myofiber may limit the ability to exercise. This is supported by findings in chronic heart failure (CHF) and chronic obstructive pulmonary disease (COPD) patients who display lower peak oxygen consumption, reduced skeletal muscle capillary density, and capillary-to-fiber ratio, as well as lower skeletal muscle oxygen tension (16, 26). In addition, inhibited vascular endothelial growth factor (VEGF) signaling has been reported in locomotor skeletal muscle of such patients (4, 8, 23). VEGF is important in both muscle capillary maintenance and in the angiogenic response to exercise training (36, 43). This makes VEGF and other angiogenic factors, linked with improving oxygen metabolism, attractive targets for the development of therapeutics to treat peripheral vascular dysfunction. However, VEGF is expressed by several cell types within muscle, including the myofiber itself, endothelial cells, satellite cells, and white blood cells.

The formation of new capillaries within the muscle depends on dynamic cell-cell interactions (2, 12, 15). An increase in the number of satellite cells parallels the formation of new capillaries in response to exercise training (13). These myogenic precursor cells are in turn supported by factors secreted by macrophages in contact with the capillary endothelial cells (12) and, a shift in the balance of pro-arteriogenic (M2) versus inflammatory (M1) macrophages has been suggested to play a role in the formation of collateral arteries in ischemic muscle (41). In endothelial cells, VEGF is regulated by an autocrine mechanism and functions to preserve vascular integrity (prevent endothelial cell apoptosis) (27, 32). In several nonskeletal muscle organs analyzed to date, endothelium-expressed VEGF does not appear to be essential for maintaining capillary number (27). It is thus still unclear which VEGF-producing cell types in muscle are essential for regulating vascular structure. This is despite the fact that myofiber-expressed VEGF represents 80–90% of the VEGF content in hindlimb skeletal muscle. Knowing the cellular origin of VEGF, essential to maintaining capillarity, is important for targeting therapeutic strategies to the responsible cell type.

Previous studies from our laboratory suggest that VEGF has an important role in maintaining the number of skeletal muscle capillaries under sedentary (cage-confined) conditions (43). The injection of an AAV/Cre virus into a localized region of VEGF*loxP* mouse gastrocnemius resulted in a loss of two-thirds of the capillaries in the transfected region (43), but this approach deletes VEGF from all cell types (including muscle fibers and capillary endothelial cells) in that region. In a subsequent study, using a Cre/LoxP cross-breeding strategy to delete VEGF just within the mouse myofiber, its lifelong deletion in both cardiac and skeletal myofibers resulted in substantial reduction in the number of capillaries measured in the heart and gastrocnemius. These mice performed very poorly when exercise tested on a treadmill (35) and were unable to form new skeletal muscle capillaries or improve overall exercise capacity with aerobic training (36). However, in these studies, it remains unclear whether the poor outcomes in adulthood are due to improper vascular system development during late embryogenesis and/or early postnatal life (35). This is important clinically, because in many human diseases of relevance, such as COPD, the vascular system likely develops normally, with disease appearing later in adulthood.

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In the present study we hypothesized that VEGF expressed by skeletal myofibers, which have developed a normal vascular system, is essential for the angiogenic response to exercise training. Furthermore, it is predicted that an inability to increase the number of capillaries surrounding myofibers would limit O_2 availability to mitochondria and prevent improvement of oxidative metabolism and exercise capacity that normally occurs in response to training. To test this hypothesis, the VEGF gene was conditionally deleted in skeletal myofibers of adult VEGF*LoxP* mice via a tamoxifen-inducible HSA-Cre-ER^{T2} system (40). Wild-type (WT) and skeletal myofiber VEGF-deficient mice (skmVEGF−/−) were subjected to a progressive treadmill exercise training schedule for 8 wk. Vascular structure (capillarity and arteriogenesis), oxidative and glycolytic enzyme capacity, fiber composition, and muscle contractile function were evaluated and related to the exercise capacity of WT and skmVEGF−/− mice.

MATERIALS AND METHODS

Experimental animals. Mice homozygous for the VEGF*Lox*P transgene (19) and heterozygous for HSA-Cre-ER^{T2} (40) were maintained for this study. Mice were housed in standard mouse cages in a pathogen-free vivarium, with a 12:12-h light-dark cycle, and fed a standard mouse diet (Harlan Tekland 8604, Madison, WI) and water ad libitum. This study was approved by the University of California, San Diego, Animal Care and Use Committee and conducted in accordance to guidelines outlined by the *Guide for the Care and Use of Laboratory Animals* (National Insitutes of Health).

Genotyping. The presence or absence of the HSA-Cre-ER^{T2} transgene in each mouse was determined by PCR analysis using tail DNA and forward 5′-CTAGAGCCTGTTTTGCACGTTC-3′ and reverse primers 5′-TGCAAGTTGAATAACCGGAAA-3′ under the following conditions: one 2-min polymerase activation incubation at 95°C, 35 cycles of 30 s denaturation at 94°C, 30 s annealing at 52.1°C, 60 s elongation at 72°C, followed by one 8-min elongation period at 72°C.

Experimental design. HSA-Cre-ER^{T2}-positive (pre-skmVEGF−/−) and control littermates (VEGF+/+) were randomly assigned to exercise-trained (EX) or cage-confined untrained (UN) groups for a total of four experimental groups (n = 9–10 mice per group). Adult (4 mo old) pre-skmVEGF−/− and VEGF +/+ mice were weighed and treadmill-exercise tested for maximal speed and endurance on 2 consecutive days. All mice then received tamoxifen from *day 0–4* (1 mg/day for 5 days ip). On *day 21*, post-tamoxifen mice were retested for speed and endurance. skmVEGF−/− EX and VEGF+/+ EX mice were exercise trained or remained cage confined for 8 wk. Maximal speed and endurance were retested upon completion of this 8-wk period. Mice were again weighed. Soleus, plantaris, gastrocnemius, and extensor digitorum longus (EDL) muscles from one leg were isolated, weighed, frozen in liquid nitrogen, and stored at −80°C for analysis or mounted for preparation of cryosections.

Exercise testing and training. Twenty-four to seventy-two hours before exercise testing, mice were familiarized on the treadmill (model CL-4, Omnitech, Columbus, OH) by running for 10 min at 10–15 cm/s on a 10° incline. Maximal speed was measured by running mice at 33 cm/s for 1 min and increasing the speed by 3–4 cm/s each minute until exhaustion. Endurance capacity was determined by running mice at 33 cm/s (50% of skmVEGF−/− average maximal speed) until exhaustion. Mice were deemed exhausted at the point they could not remain on the treadmill and sat on shock grid (≤0.3 milliamps) for 10 s. Exercise training consisted of 1 h of treadmill running 5 days per week, increasing speed from 28 to 34 cm/s over 8 wk. All mice groups received the same absolute speed and duration throughout 8 wk of exercise training.

Ex vivo muscle contractile function and fatigue resistance. Contractile properties of the EDL and soleus were measured ex vivo as previously described (33, 44). EDL and soleus mounted in experimental chambers (800MS, Danish Myo Technology, Aarhus, Denmark) were perfused with Tyrode solution (in mM: 121 NaCl, 5 KCl, 0.4 NaH_2PO_4, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 24 $NaHCO_3$, 5.5 glucose, and 0.1 EGTA) continuously bubbled with 95% O_2-5% CO_2 (pH 7.4, 22°C). Muscles were electrically stimulated (S88X stimulator, Grass Technologies) using square-wave pulses (16 V; EDL: 300 ms train duration, 0.5 ms pulse duration; soleus: 500 ms train duration, 0.5 ms pulse duration) with single twitches to set the length for maximal twitch tension (L_0). After 15 min, contractile function was evaluated by stimulating the muscles at different frequencies (EDL muscle: 1–150 Hz; soleus: 1–120 Hz) with 100-s intervals between contractions. After the force-frequency protocol, muscles rested for 10 min. Fatigue resistance was determined by a series of repeated tetanic contractions at a frequency that evoked near-maximal tetanic tension development (70 Hz for EDL and 50 Hz for soleus) with increasing train frequency (1 contraction each 8, 4, 3, 2, and 1 s) each minute (for EDL) or every 2 min (for soleus) until the initial force decreased to 50% (fatigue point). Tension development (in mN) was normalized with respect to the muscle cross-sectional area (mN/mm^2). Cross-sectional area was calculated dividing muscle mass (mg) by the product of L_o (mm) times muscle density (1.06 mg/mm^3).

VEGF protein levels. VEGF protein levels were measured by ELISA (VEGF Mouse ELISA, R&D Systems, La Jolla, CA) and normalized to total protein levels (Bio-Rad DC protein assay).

Skeletal muscle morphology and immunohistochemistry. Capillaries and fibers in 10-μm cryosections were detected using the Capillary Lead-ATPase method (39). Images were captured using a Hamamatsu Nanozoomer Slide Scanning System. Total capillary number, total fiber number, fiber areas, and type were calculated using ImageJ software. 5′-Bromodeoxycytidine (BrdU) was injected (50 mg/kg ip) daily for 6 consecutive days before animals were euthanized. For immunohistochemistry 10-ìm cryosections were fixed with 4% paraformaldehyde for 10 min at room temperature, rinsed with PBS, and incubated overnight at 4°C with the following primary antibody concentrations to detect proliferating cells, fibroblasts, and smooth muscle cells:BrdU mouse mAB (0.2 mg/ml, B35128, Life Technologies), vimentin rabbit mAB (1:200, no. 5741, Cell Signaling), α-smooth muscle antibody (1:200, A2547, Sigma), and F4/F80+ (1:100, no. 14-5989, eBiosceince, San Diego, CA). BrDU sections were treated with 2 M HCl at 56°C for 30 min and washed with PBS before primary antibody incubation. Signals were detected with anti-mouse and anti-rat AlexaFlour 488 or anti-rabbit AlexaFluor 546 (Invitrogen, Molecular Probes, Carlsbad, CA) and mounted with ProLong Gold antifade reagent with 4′,6-diamidino-2-phenylindole (DAPI, P-36931; Invitrogen, Carlsbad, CA). Images were collected with an Olympus FV100 confocal microscope.

Metabolic enzyme analysis. Metabolic enzyme activities [expressed in units (U) of catalyzing mmol substrate·mg tissue^{-1}·min^{-1} in muscle homogenates] were measured at room temperature as previously described (44).

Statistical analysis. A two-way ANOVA was used to detect difference between exercise condition and genotype. A Tukey post hoc test was used to analyze specific differences between the four experimental groups. $P < 0.05$ is considered significant. All data are presented as means ± SE.

RESULTS

Conditional ablation of the VEGF gene in adult skeletal myofibers. VEGF protein levels, measured at the end of the training period, were decreased in the soleus, plantaris, gastrocnemius, and EDL by 80, 86, 85, and 76% ($P < 0.01$), respectively, in untrained skmVEGF−/− mice compared with WT, untrained VEGF+/+ mice. After 8 wk of progressive

treadmill training was completed, VEGF protein levels were higher in the soleus, plantaris, gastrocnemius, and EDL by 23, 21, 19, and 25% ($P < 0.01$), respectively, in WT exercise-trained mice (VEGF+/+ EX mice) compared with untrained, WT mice. No differences in VEGF protein levels were observed in the soleus, plantaris, gastrocnemius, or EDL in skmVEGF−/− mice after 8 wk of training (Fig. 1) compared with untrained skmVEGF−/− mice.

Exercise capacity in response to treadmill exercise training. Maximal running speed was unchanged from pre-tamoxifen level in VEGF+/+ and skmVEGF−/− mice 21 days after administration of tamoxifen (tested before exercise training). Maximal speed increased by 18% in VEGF+/+ mice after 8 wk of training (VEGF+/+ UN, 60.11 ± 1.5 cm/s; VEGF+/+ EX, 69.8 ± 1.1 cm/s; $P < 0.01$). No difference in maximal speed of skmVEGF−/− mice was observed after training (skmVEGF−/− UN, 57.4 ± 1.1 cm/s; skmVEGF−/− EX, 56.2 ± 4.4 cm/s) (Fig. 2*A*). On *day 22* (but not at the later 11-wk time point) post-tamoxifen, skmVEGF−/− mice exhibited a 30% reduction in the time to exhaustion compared with untrained, VEGF+/+ mice (skmVEGF−/−, 60.2 ± 6.1 min; VEGF+/+, 86.1 ± 6.6 min; $P < 0.01$). However, the skmVEGF−/− EX group did not improve endurance capacity with exercise training. WT VEGF+/+ mice increased the time to reach exhaustion by 99% (time to exhaustion: VEGF+/+ UN, 84.1 ± 7.8 min, VEGF +/+ EX, 167.3 ± 5.1 min, $P < 0.01$) (Fig. 2*B*).

Body weights and skeletal muscle mass. Body mass decreased by 11.2% in VEGF+/+ mice in response to 8 wk of exercise training (VEGF +/+ UN, 24.43 ± 0.70 g; VEGF +/+ EX, 21.68 ± 0.27 g; $P < 0.01$) but did not change in skmVEGF−/− mice. No change in muscle mass-to-body weight ratio (mg/g) was observed in the soleus, plantaris, gastrocnemius, or EDL between groups (Table 1).

Ex vivo muscle contractile function. Maximal tetanic force (Fig. 3, *A* and *C*) and the force-frequency relationship (Fig. 3, *B* and *D*) were not affected by exercise training in the soleus and EDL. Maximal tetanic force was reduced in the soleus muscle of skmVEGF−/− EX mice, yet this did not change the force-frequency relationship (Fig. 3, *A* and *B*). EDL isolated from exercise-trained skmVEGF−/− revealed an increase in the time to fatigue, or improved fatigue resistance, compared with the other three groups (Fig. 3*E*). Exercise training or VEGF gene deletion did not alter isolated soleus fatigue resistance (Fig. 3*E*).

Angiogenic response to exercise training. After training, VEGF+/+ EX mice increased capillary-to-fiber ratio in the plantaris (40%) and gastrocnemius (43%). Capillary density in VEGF+/+ mice also increased in the soleus (27%), plantaris (38%), and gastrocnemius (49%). Fiber area was unchanged between untrained and exercise-trained VEGF+/+ mice. In the skmVEGF−/− mice, the only muscle to demonstrate an angiogenic response to training was the plantaris. Capillary-to-fiber ratio and capillary density were increased by 18% and 25%, respectively, in the plantaris of skmVEGF−/− mice. skmVEGF−/− gastrocnemius mean fiber area was reduced by 18% and accompanied by a 6% increase in capillary density (Table 2).

Arteriogenesis and skeletal muscle remodeling. The number of α-smooth muscle actin (αSMA+) arteries per gastrocnemius complex cross-sectional area increased with both exercise training and VEGF gene ablation. There was no interaction between these two conditions (Fig. 4, *B* and *D*). Analysis of arterial size revealed more arteries with diameters in the 30- to 60-μm range in skmVEGF−/− mice, both with and without exercise training, compared with the VEGF+/+ groups.(Fig. 4, *B* and *E*). BrdU-positive cells were present in areas of increased vimentin+ fibroblasts (Fig. 4*A*). Quantification of

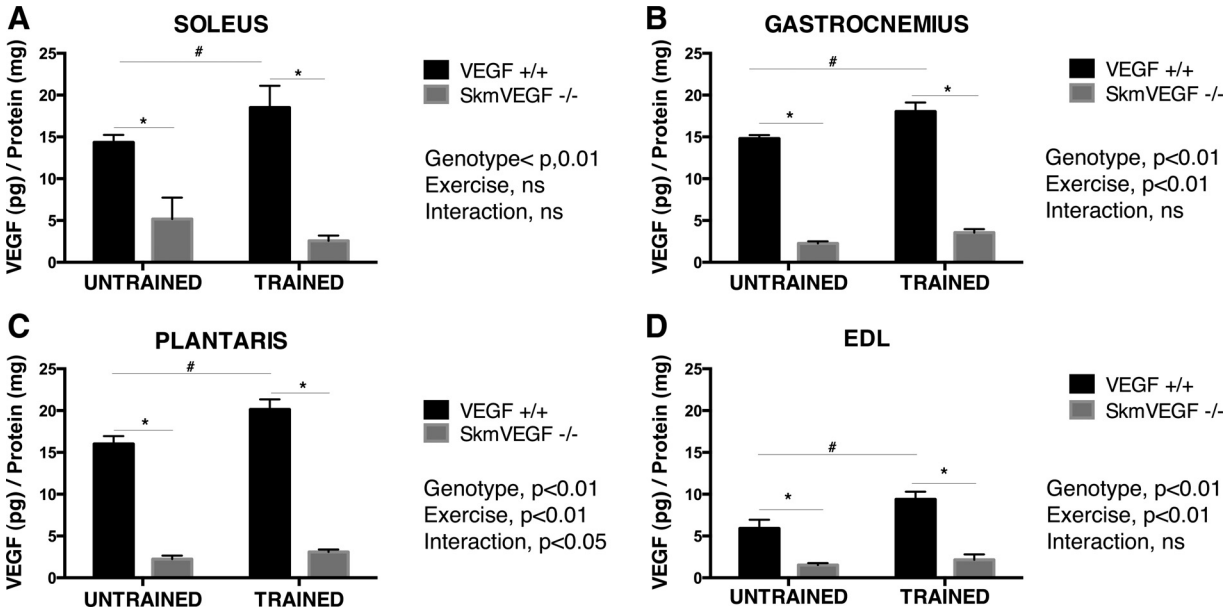

Fig. 1. VEGF levels in hindlimb muscles from wild-type and skeletal myofiber VEGF gene-ablated mice. VEGF protein in the soleus (*A*), gastrocnemius (*B*), plantaris (*C*), and extensor digitorum longus (EDL) from VEGF+/+ and skeletal myofiber-specific VEGF gene-deleted (skmVEGF−/−) mice (*D*) that were either exercise trained (TRAINED) or remained cage-confined (UNTRAINED). Values are means ± SE, $P < 0.01$. *P indicates a significant difference between genotype within the same UNTRAINED or TRAINED group, #P indicates a significant difference between exercise condition within the same genotype.

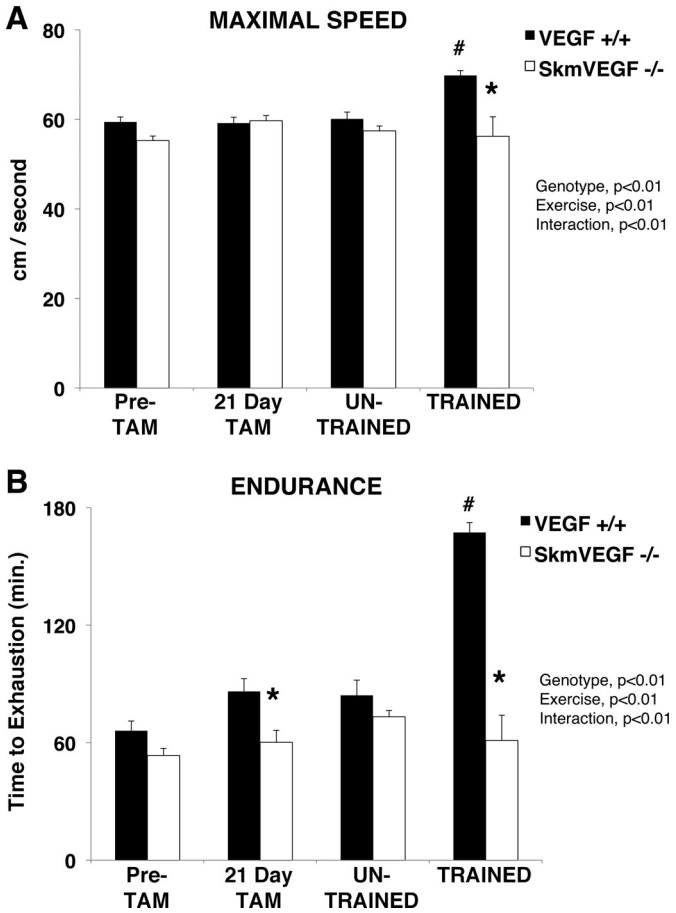

Fig. 2. Treadmill exercise capacity. Maximal speed (*A*) and endurance (*B*) were tested in VEGF+/+ and skmVEGF−/− mice at the following time points: pre-tamoxifen (TAM) (*day 0*), 21 days post-tamoxifen to conditionally delete the VEGF gene, and 8 wk after cage confinement or exercise training (*day 78*). Values are means ± SE, $P < 0.01$. *P indicates a significant difference between genotype within the same untrained or exercissed group, #P indicates a significant difference after exercise training within the same genotype (n = 9–10).

BrDU+ cell per myofiber revealed increases in the gastrocnemius of UN and EX skmVEGF−/− mice compared with UN and EX VEGF+/+ groups, respectively (skmVEGF−/− UN, 41 ± 2%; VEGF+/+ UN, 11 ± 2%; $P < 0.01$. skmVEGF−/− EX, 50 ± 3%; VEGF+/+ EX, 29 ± 2%; $P < 0.01$). Exercise training, alone, also increased BrdU incorporation in VEGF+/+ muscles (Table 3). Vimentin-positive cells around the myofibers and in vessel walls also increased with VEGF gene deletion and were augmented with exercise training. Vimentin+ cells per myofiber increased in both UN and EX skmVEGF−/− mice compared with VEGF+/+ UN and VEGF+/+ EX groups in the gastrocnemius (skmVEGF−/− UN, 51 ± 7%; VEFG+/+ UN, 26 ± 4%, $P < 0.01$. skmVEGF−/− EX, 87 ± 1%; VEGF+/+ EX, 35 ± 4%; $P < 0.01$). Gastrocnemius sections from exercise-trained skmVEGF−/− mice displayed clusters of numerous vimentin+/BrdU+ cells around vessels (Table 3). In addition, numerous inflammatory macrophages (F4/80+ cells) were present in untrained skmVEGF−/− (Fig. 4*C*).

Metabolic response to exercise training. Citrate synthase (CS) activity increased 49%, 44%, 54%, and 13% in the soleus, plantaris, gastrocnemius, and EDL, respectively, of VEGF+/+ mice after training. skmVEGF−/− mice increased CS activity by only 21%, 22%, and 28% in the soleus, plantaris, and gastrocnemius, respectively. β-Hydroxyacyl CoA dehydrogenase (β-HAD) activity, a marker for fatty acid β-oxidation, increased in the soleus (36%), plantaris (29%), gastrocnemius (28%), and EDL (14%) ($P < 0.01$) in VEGF+/+ mice with training. β-HAD activity was not increased in response to training in skmVEGF−/− mice but was higher in the plantaris and gastrocnemius in the untrained condition compared with WT mice. Activity of the glycolytic enzyme phosphofructokinase (PFK) increased in both VEGF+/+ and skmVEGF−/− mice after training in the soleus (VEGF+/+, 17%; skmVEGF−/−, 11%), plantaris (VEGF+/+, 21%; skmVEGF−/−, 21%), gastrocnemius (VEGF+/+, 21%; skmVEGF−/−, 19%), and EDL (VEGF+/+, 20%; skmVEGF−/−, 22%) (Fig. 5). Fiber-type composition was unchanged in the soleus, plantaris, or gastrocnemius. However, the EDL showed an increase ($P < 0.01$) in type IIa fibers and reduction in IIb fibers in UN skmVEGF−/− mice compared with control UN VEGF+/+ mice (UN skmVEGF−/−, 11.6 ± 1.2%; UN VEGF+/+, 6.4 ± 0.6%). Eight weeks of exercise training increased ($P < 0.01$) type IIa fibers in both skmVEGF−/− and VEGF+/+ mice (skmVEGF−/− EX, 13.1 ± 1.4%; VEGF+/+ EX, 14.1 ± 2.1%) (data not shown).

DISCUSSION

This study suggests that VEGF expressed by skeletal myofibers is essential in adult mice to improve exercise capacity in response to an 8-wk treadmill running program. The angiogenic adaptation to treadmill running was completely or partially inhibited depending on muscle type. Furthermore, the training-induced increase in oxidative metabolic capacity, namely CS and β-HAD activity, was attenuated in skmVEGF−/− mice. In contrast, the glycolytic response to exercise training (PFK enzyme activity) was not altered, and

Table 1. *Body weights and skeletal muscle mass*

	VEGF+/+		skmVEGF−/−	
	Untrained (n = 9)	Exercise Trained (n = 10)	Untrained (n = 9)	Exercise Trained (n = 10)
Body mass, g	24.4 ± 0.7	21.7 ± 0.2*	23.0 ± 0.7	22.2 ± 0.4
Soleus, mg/g	0.31 ± 0.01	0.31 ± 0.01	0.30 ± 0.01	0.29 ± 0.01
Plantaris, mg/g	0.74 ± 0.08	0.77 ± 0.06	0.68 ± 0.05	0.68 ± 0.03
Gastrocnemius, mg/g	4.08 ± 0.19	4.57 ± 0.23	3.73 ± 0.23	4.07 ± 0.16
EDL, mg/g	0.32 ± 0.01	0.36 ± 0.02	0.33 ± 0.01	0.30 ± 0.03

Values are means ± SE; n is number of mice. skmVEGF−/−, skeletal myofiber-specific vascular endothelial growth factor (VEGF) gene-deleted mice; EDL, Extensor digitorum longus. *P indicates a significant difference after exercise training within the same genotype (n = 9–10).

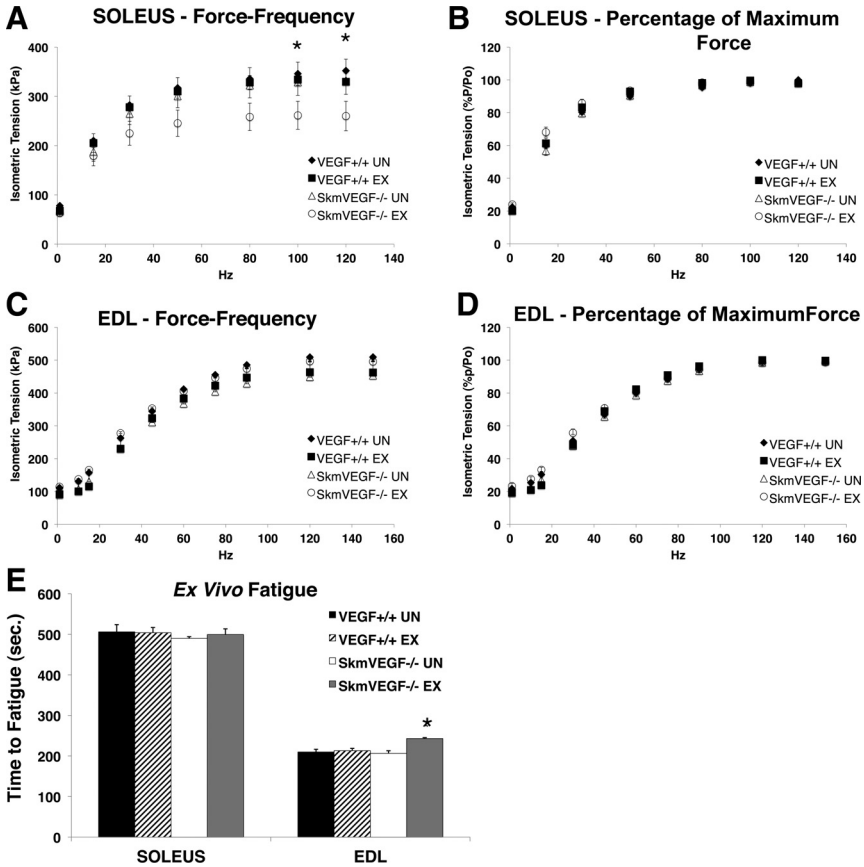

Fig. 3. Ex vivo soleus and EDL contractile muscle function. Total (*A*, *C*) and relative (*B*, *D*) force-frequency measurements from the soleus (*A* and *B*) and EDL (*C* and *D*). Values are means ± SE, $n = 6–9$ $P < .05$. **P* indicating significant difference between skmVEGF−/− exercised mice and all groups. *E*: time to fatigue (seconds) was recorded in soleus and EDL. Values are the means ± SE, $n = 6–9$ *Difference between the skmVEGF−/− exercised mice and all other groups ($P < 0.01$).

skeletal muscle contractile properties (analyzed ex vivo) were not compromised. Interestingly, there was an increase in two compensatory mechanisms, which could potentially contribute to the observed restoration of exercise capacity (after early impairment) in untrained VEGF-deficient mice. The first is a potential increase in lipid utilization, which is implied from greater β-HAD activity in skmVEGF−/− mice that were not subjected to exercise training. The second was an increase in the size and number of arteries, (i.e., arteriogenesis). Overall, these data suggest that inhibition of VEGF-dependent angiogenesis in exercise-trained mice prohibits any improvement in the integrated exercise response.

Inhibition of skeletal myofiber VEGF expression in adult mice. In this study a genetic approach was used to conditionally inhibit VEGF expression in mature skeletal myofibers of adult mice. This mouse model was suitable for our study as VEGF gene deletion was solely targeted to skeletal muscle myofibers, such that other cell types within muscle were

Table 2. *Skeletal muscle morphometry*

	VEGF+/+		skmVEGF−/−	
	Untrained ($n = 9$)	Exercise Trained ($n = 9$)	Untrained ($n = 9$)	Exercise Trained ($n = 10$)
Capillary/fiber ratio				
Soleus	1.64 ± 0.06	1.82 ± 0.07	1.54 ± 0.04	1.69 ± 0.05
Plantaris	1.15 ± 0.03	1.61 ± 0.08†	1.13 ± 0.03	1.33 ± 0.04*†
Gastrocnemius.	1.14 ± 0.03	1.63 ± 0.07†	1.10 ± 0.02	1.18 ± 0.05*
EDL	1.24 ± 0.03	1.30 ± 0.02	1.12 ± 0.03	1.26 ± 0.06
Mean fiber area, μm^2				
Soleus	1,953 ± 116	1,677 ± 43	1,795 ± 63	1,831 ± 144
Plantaris	1,731 ± 63	1751 ± 88	1,895 ± 42	1,792 ± 87
Gastrocnemius	2,697 ± 78	2,601 ± 142	2,677 ± 81	2,199 ± 84*,†
EDL	1,912 ± 89	1,845 ± 125	1,936 ± 101	2,088 ± 162
Capillary density, cap/mm^2				
Soleus	861 ± 59	1,095 ± 48†	864 ± 30	968 ± 74
Plantaris	670 ± 27	926 ± 42†	599 ± 23	751 ± 35*†
Gastrocnemius	426 ± 12	635 ± 29†	413 ± 14	536 ± 17*†
EDL	661 ± 37	723 ± 47	594 ± 38	629 ± 46

Values are means ± SE; *n* is number of mice. $P < 0.01$. **P* indicates a significant difference between genotype within the same untrained or exercised group; †*P* indicates a significant difference after exercise training within the same genotype.

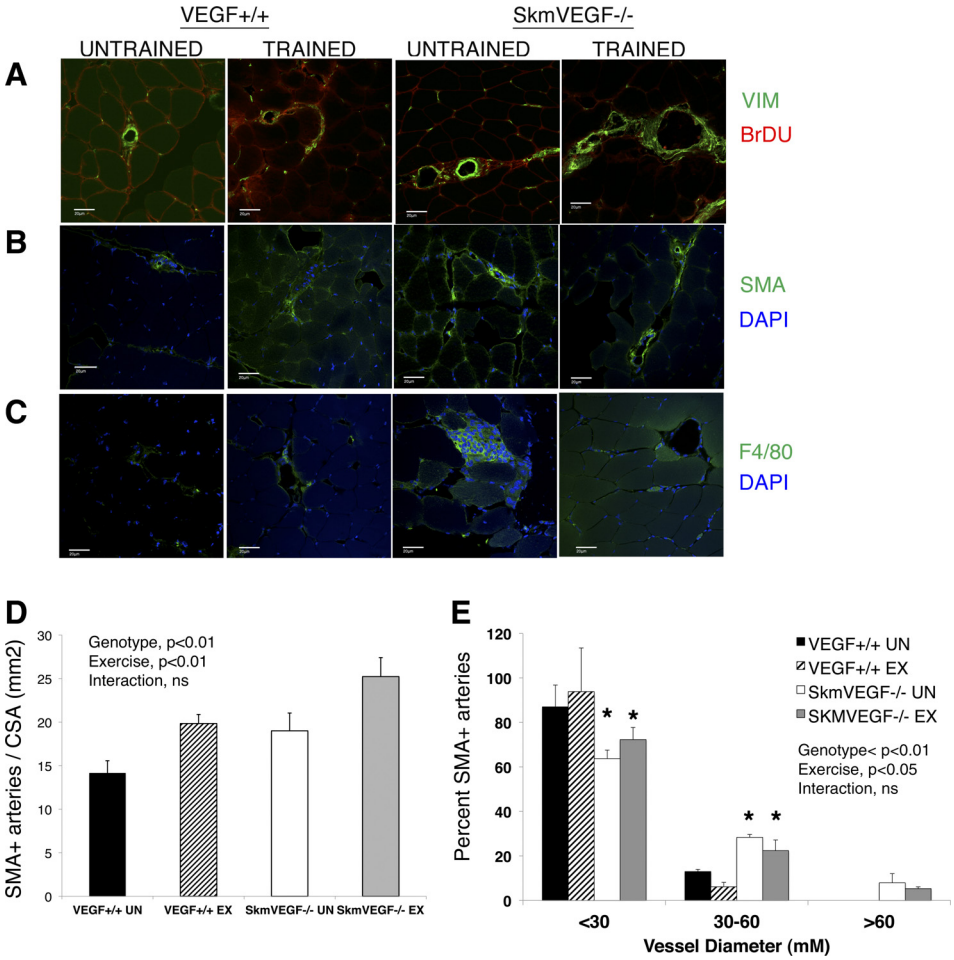

Fig. 4. Vascular remodeling in skmVEGF−/− mice. Confocal images of 5′-bromodeoxycytidine (BrdU)+ and vimentin+ (VIM) cells (*A*), α-smooth muscle actin+ (SMA) vessels (*B*), and F4/F80+ (F4/80) macrophages (*C*) in the gastrocnemius. The total number of SMA+ arteries per cross-sectional area (mm^2) in the gastrocnemius were counted in each group (*D*). The distribution of arterial diameters (μm) are represented as the percentage of the total number counted (*E*). DAPI, 4′,6-diamidino-2-phenylindole. Values are means ± SE, $P < 0.01$. *P indicates a significant difference between genotype within the same UN or EX group, #P indicates a significant difference after exercise training within the same genotype ($n = 4$).

spared. Furthermore, these mice were able to develop a normal vascular system before inducing VEGF gene deletion. This situation more closely models chronic and age-related conditions found in older humans. In WT mice, exercise training elevated VEGF protein levels, but VEGF levels were neither restored nor increased with exercise training in skmVEGF−/− mice. The exercise-induced increase in VEGF in the control group is consistent the study of Waters et al. (49), who reported increased VEGF levels after 3 and 7 days of voluntary wheel running. Thus the present study shows skeletal muscles uphold this elevated VEGF level with training over an extended 8-wk period.

Progressive intensity training protocol. Gute et al. (21, 22) have shown that the intensity of exercise training can have a significant effect on the regional angiogenic response in rat skeletal muscle. Therefore, a training protocol was designed to subject each mouse group to the same absolute exercise stimuli. To accomplish this, a group of skmVEGF−/− mice initially ran at ~50% of their maximal speed for 1 h each day, and for each mouse, we attempted to increase speed each week. WT mice exercised at the same absolute speed and duration as the skmVEGF−/− mice. Since WT mice did produce an angiogenic response, all the mice trained at a sufficient intensity to elicit angiogenesis. This is despite the fact that the relative training intensity for VEGF+/+ mice was less then the relative training intensity for the skmVEGF−/− mice. Thus any differences observed between trained mice can be attributed to VEGF gene deletion and not to differences between relative training intensities.

Inhibition of the angiogenic response to exercise training. As we originally hypothesized, the expression of VEGF by skeletal myofibers is necessary to increase the capillary-to-fiber

Table 3. *Proliferating cells and fibroblasts in the gastrocnemius from wild-type and skmVEGF−/− mice*

	VEGF+/+		skmVEGF−/−	
	Untrained ($n = 4$)	Exercise Trained ($n = 4$)	Untrained ($n = 4$)	Exercise Trained ($n = 4$)
BrdU+ cells/fiber	0.11 + 0.02	0.29 + 0.02†	0.41 + 0.02*	0.50 + 0.03*
Vimentin+ cells/fiber	0.26 + 0.04	0.35 + 0.04	0.51 + 0.07*	0.87 + 0.01*†
Vimentin+ vessels/area, mm^2	26.6 + 2.7	37.8 + 5.8	91.9 + 6.4*	155.2 + 9.5*†

Values are means ± SE; n is number of mice. BrdU, 5′-bromodeoxycytidine. $P < 0.01$. *P indicates a significant difference between genotype within the same untrained or exercised group; †P indicates a significant difference after exercise training within the same genotype.

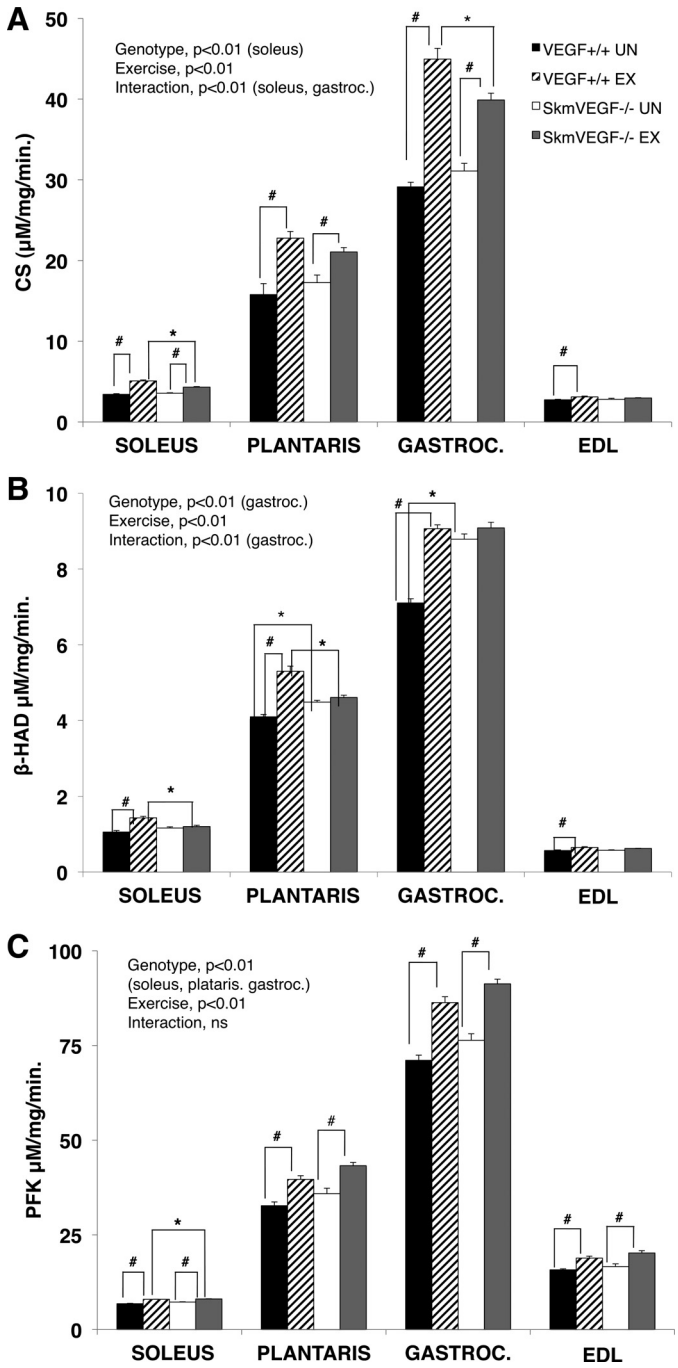

Fig. 5. Metabolic enzyme activity. Citrate synthase (CS) (*A*), β-hydroxyacyl CoA dehydrogenase (β-HAD) (*B*), and phosphofructokinase (PFK) (*C*) activites ($\mu mol \cdot mg^{-1} \cdot min^{-1}$) in the soleus, plantaris, gastrocnemius, and EDL are shown. Values are means ± SE, $P < 0.01$. *P indicates a significant difference between genotype within the same unexercised or exercised group, #P indicates a significant difference after exercise training within the same genotype (n = 4–9).

ratio with exercise training (Table 2). Interestingly, different angiogenic responses were observed in different muscle types. Exercise training and gene deletion had no effect on the capillary-to-fiber ratio in the oxidative, soleus, as well as the glycolytic, EDL. In the mixed, gastrocnemius, which is the largest hindlimb muscle and the main contributing muscle for running (7), exercise training also did not elicit an angiogenic response in skmVEGF−/− mice. In WT mice the expected training-induced increase in gastrocnemius capillary number was observed. However, in the plantaris, another mixed fiber-type muscle, but with a higher number of oxidative fibers compared with the gastrocnemius, a limited number of capillaries were formed in response to exercise training in skmVEGF−/− mice.

While the mechanism for the VEGF-independent angiogenic response in the plantaris is unknown, it may stem from a number of factors: *1*) VEGF expressed by other cells, such as endothelial cells, satellite cells, or macrophages may contribute to the observed response (1, 12, 27); and *2*) other angiogenic or anti-angiogenic factors such as angiopoietin 1, (1, 46), placental growth factor (PGF), platelet-derived growth factor (PDGF), and fibroblast growth factor-2 (FGF2) and thrombospondin, which act in a synergy with VEGF, and may play a more prominent role to compensate for the loss of VEGF (6, 10, 11, 13, 20, 29, 34). While these mechanisms may account for the modest angiogenic response in the plantaris of skmVEGF−/− mice, these data demonstrate the necessity of myofiber-expressed VEGF to achieve a full exercise-induced angiogenic response. Furthermore, the limited angiogenic response present in skmVEGF−/− mice was insufficient for improving exercise capacity.

These data collected in skeletal myofiber VEGF-ablated mouse model are very similar to the situation in COPD patients. A recent study investigating the response COPD patients to exercise training found that the angiogenic adaptation was greatly reduced and correlated with symptom-limited oxygen consumption (20). On possibility is that VEGF expressed by mature myofibers is important for regulating satellite cells associated with skeletal myofibers. Christov et al. (13) have reported that the number of these adult stem cells, satellite cells, increases in parallel with the formation of new capillaries in response to exercise training in humans. Interestingly, in addition to playing a role in repairing damage, myofibers satellite cells have the potential to signal endothelial cells to initiate the formation of new capillaries (2, 18). This has been demonstrated to occur through both hypoxia and VEGF-dependent mechanisms in vitro coculture systems of satellite cells and endothelial cells. Furthermore, satellite cell-mediated VEGF expression and angiogenesis is compromised when muscle dysfunction is present due to age or muscular dystrophy (38). In COPD a greater number of senescent satellite cells, with limited muscle regenerative capacity, are present in advanced stages characterized by muscle wasting (31, 45). Further studies will be required to determine the potential mechanisms that may limit the training-induced angiogenic potential of satellite cells in humans exhibiting chronic exercise intolerance.

Maintenance of capillaries in the untrained state. Capillary number in sedentary skmVEGF−/− mice was unaltered compared with WT untrained mice. This contrasts the study by Tang et al. (43) in which an AAV/Cre-mediated VEGF gene ablation in a limited region of the gastrocnemius resulted in capillary regression. One possible explanation is that the AAV/Cre (43) was more efficient at reducing VEGF levels in the 30–50 contiguous fibers per muscle that were infected. Alternatively, simultaneous reduction in VEGF expressed by more than one cell population within skeletal muscle (i.e., endothe-

lial cell, smooth muscle cells, fibroblasts) may be necessary for capillary regression to occur.

Restoration of exercise capacity in untrained mice. Endurance capacity is initially impaired in skmVEGF−/− mice before exercise training. Previous studies have shown that at this early time point, contraction-induced blood flow and fatigue resistance measured in situ (with an intact blood supply) is diminished (A. Knapp, unpublished observations, 2013). In the current study VEGF levels remained low in skmVEGF−/− mice for 11 wk post-gene deletion. However, there was no longer a detectable difference in endurance capacity between untrained, WT and untrained, skeletal myofiber VEGF gene-deleted mice 11 wk after initiating the gene deletion. Two observed compensatory changes in the muscle could contribute to this restoration in exercise capacity in the untrained state. First, there may be an increased utilization of lipids, as evident from enhanced β-HAD activity. A similar compensation in metabolic enzyme activity has been reported in both mice with lifelong, myofiber VEGF gene ablation [MckCre × VEGF*LoxP* mice (36)] and patients with peripheral arterial disease (PAD) (9, 24, 30, 48). However, it should be noted that mice with life-long myofiber VEGF gene deletion and patients with PAD exhibit poor exercise capacity. The second adaptation, which also occurs in PAD patients, is arteriolization of the small vessels or capillaries independent of exercise training (5, 14, 42) (Fig. 3). Takeda et al. (41) have recently shown Phd2 haplo-deficient mice form collateral arteries, which function to preserve limb perfusion and exercise capacity when challenged with an ischemic insult.

The findings of increased arteriogenesis and maintenance of capillaries in the gastrocnemius of untrained skmVEGF−/− mice are in contrast to VEGF receptor inhibition studies. Inhibition of VEGF signaling through VEGF neutralizing antibodies, receptor blockade, or soluble VEGF traps have all been shown to prevent the formation of collateral vessels in ischemic hindlimb muscles (25, 28, 47), and in some of these studies exercise-induced angiogenesis was partially inhibited (25, 28). More recently, Moraes et al. (32) have shown that inhibition of VEGF signaling, through endothelium-targeted synectin gene deletion, is essential for ischemia-induced arteriogenesis. Thus collectively these studies suggest that VEGF signaling in endothelial cells may be important for stimulating arteriogenesis in hindlimb ischemia. In contrast VEGF expressed by myofibers and possibly smooth muscle cells may be important for limiting arteriogenesis. Alternatively, the signals regulating the formation of arteries and capillaries in pathological ischemia may differ from those in response to a normal physiological stimulus, exercise training.

Skeletal muscle contractile properties in skmVEGF−/− mice. Improved contractile function and a transition to more oxidative fiber types are adaptive responses to exercise training in many species (17, 21, 22). In the present study, WT mice did not show an increase in the percentage of oxidative type I or IIa fibers in the soleus, plantaris, or gastrocnemius muscles and showed only a minor transition to more oxidative type IIa fibers in the EDL muscle. These data suggest that the training paradigm (e.g., intensity and/or duration) was likely not intense enough for an adaptation in fiber composition. Furthermore, myofiber-deficient VEGF mice did not display any major impairment in ex vivo muscle contractile function. In fact, fatigue resistance was slightly improved in the EDL of exercise-trained skmVEGF−/− mice for reasons that remain unclear. The EDL muscles showed a slight shift from type IIb to IIa fibers with training, but this occurred in both WT and skmVEGF−/− mice (37). Since the mechanism of muscle fatigue is multifactorial [e.g., Ca^{2+}-handling changes, myofilament dysfunction, energy substrate depletion, metabolite accumulation (3)], these data suggest that muscles from trained skmVEGF−/− mice may have intracellularly compensated for the inability to increase capillaries with training.

Perspectives and Significance

This study establishes the critical role of myofiber-expressed VEGF in achieving the full angiogenic response to exercise training and in improving overall exercise capacity. While a number of physiological compensations are observed in skmVEGF−/− mice, including arteriogenesis, improved EDL fatigue resistance, and enhanced oxidative metabolism, none of these mechanisms translated into an improvement in maximal running speed or endurance. Thus these data indicate that VEGF expressed by skeletal myofibers is necessary for: *1*) improving exercise capacity in response to training; and *2*) regulating the exercise-induced angiogenic response in locomotor muscles. These findings highlight the importance of skeletal muscle VEGF in stimulating capillary growth during exercise training.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: H.D., L.N., D.M., and E.C.B. performed experiments; H.D., L.N., P.D.W., M.C.H., and E.C.B. analyzed data; H.D., L.N., P.D.W., M.C.H., D.M., and E.C.B. interpreted results of experiments; H.D., L.N., and E.C.B. prepared figures; H.D. and E.C.B. drafted manuscript; H.D., L.N., P.D.W., M.C.H., D.M., and E.C.B. edited and revised manuscript; H.D., L.N., P.D.W., M.C.H., D.M., and E.C.B. approved final version of manuscript; E.C.B. conception and design of research.

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