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Selective Life-Long Skeletal Myofiber—Targeted VEGF Gene Ablation Impairs Exercise Capacity in Adult Mice

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Exercise is dependent on adequate oxygen supply for mitochondrial respiration in both cardiac and locomotor muscle. To determine whether skeletal myofiber VEGF is critical for regulating exercise capacity, independent of VEGF function in the heart, ablation of the VEGF gene was targeted to skeletal myofibers (skmVEGF−/−) during embryogenesis (~ E9.5), leaving intact VEGF expression by all other cells in muscle. In adult mice, VEGF levels were decreased in the soleus (by 65%), plantaris (94%), gastrocnemius (74%), EDL (99%) and diaphragm (64%) ($P < 0.0001$, each muscle). VEGF levels were unchanged in the heart. Treadmill speed (WT 86 ± 4 cm/sec, skmVEGF−/− 70 ± 5 cm/sec, $P = 0.006$) and endurance (WT 78 ± 24 min, skmVEGF−/− 18 ± 4 min, $P = 0.0004$) were severely limited in skmVEGF−/− mice in contrast to minor effect of conditional skmVEGF gene deletion in the adult. Body weight was also reduced (WT 22.8 ± 1.6 g, skmVEGF−/−, 21.1 ± 1.5, $P = 0.02$), but the muscle mass/body weight ratio was unchanged. The capillary/fiber ratio was lower in skmVEGF−/− plantaris (WT 1.51 ± 0.12, skmVEGF−/− 1.16 ± 0.20, $P = 0.01$), gastrocnemius (WT 1.61 ± 0.08, skmVEGF−/− 1.39 ± 0.08, $P = 0.01$), EDL (WT 1.36 ± 0.07, skmVEGF−/− 1.14 ± 0.13, $P = 0.03$) and diaphragm (WT 1.39 ± 0.18, skmVEGF−/− 0.79 ± 0.16, $P = 0.0001$) but, not in soleus. Cardiac function (heart rate, maximal pressure, maximal dP/dt, minimal dP/dt,) in response to dobutamine was not impaired in anesthetized skmVEGF−/− mice. Isolated soleus and EDL fatigue times were 16% and 20% ($P < 0.02$) longer, respectively, in skmVEGF−/− mice than the WT group. These data suggest that skeletal myofiber VEGF expressed during development is necessary to establish capillary networks that allow maximal exercise capacity.

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Older individuals and those that suffer from chronic diseases, such as chronic obstructive pulmonary disease (COPD) (Barreiro et al., 2008) and peripheral artery disease (PAD) (Rajagopalan and Mohler, 2003), often exhibit exercise limitation. One change observed in the locomotor skeletal muscle under these exercise-limiting conditions is a decrease in the expression of VEGF. Our laboratory has previously shown that life-long myofiber VEGF gene deletion in mice, simultaneously in both cardiac and skeletal myofibers (using Mck-Cre/VEGF*LoxP* cross-breeding), results in decreased cardiac and skeletal muscle capillarity, body weight and exercise capacity (Olfert et al., 2009). In addition, Giordano et al. (Giordano et al., 2001) have shown that specifically targeting VEGF gene deletion to cardiac myocytes results in diminished in vivo cardiac contractile function (under anesthesia) and fewer capillaries per cardiac myocyte. However, the consequence of cardiac-specific VEGF gene deletion for exercise capacity was not evaluated in this study. Moreover, we recently found that conditional, myofiber-specific VEGF deletion in skeletal muscles, achieved via a tamoxifen- inducible strategy initiated in adult mice, showed a modest (30%) effect on exercise capacity, but no effect on muscle capillarity in untrained mice (Delavar et al., 2014). Interestingly, using a viral vector to conditionally delete the VEGF gene in all cells (not restricted to the myofiber) in a localized region of the hind limb of adult mice results in gastrocnemius capillary regression (Tang et al., 2004). However, this direct injection approach targets only a small percentage of a single muscle and does not allow for physiological testing. Thus, based on these previous mouse models, the relative contributions of VEGF deficiency in skeletal versus cardiac myofibers to an overall limitation in running exercise capacity is unresolved. Our hypothesis is that life-long presence of skeletal myofiber-expressed VEGF is required for regulating the number of capillaries in skeletal muscle and overall exercise capacity in adult mice. This hypothesis was tested by crossing VEGF*LoxP* mice with mice that express *cre* recombinase only in skeletal myofibers under the control of the myogenin-MEF2 promoter/enhancer (Gerber et al., 1999; Li et al., 2005). Thus, VEGF gene ablation was restricted to skeletal myofibers throughout post-natal

Abbreviations: CD, capillary density; CF, capillary-to-myofiber ratio; COPD, chronic obstructive pulmonary disease; Cre, *cre* recombinase; FS, fractional shortening; FCSA, fiber cross-sectional area; HR, heart rate; IVSd, interventricular septal thickness at end-diastole; LVPWd, left ventricular posterior wall thickness at end-diastole; LVIDd, left ventricular internal diameter, diastole; LVIDs, left ventricular internal diameter, systole; Myo-Cre, *cre* recombinase expression under the control of the myogenin promoter-MEF2 enhancer construct; skmVEGF−/−, skeletal myofiber VEGF gene deletion mouse; Mean Vcf, mean velocity of circumferential fiber shortening; BW, body weight; VEGF, vascular endothelial growth factor; VEGF*LoxP*, mice whose VEGF gene is floxed with LoxP sites; WT, wild-type mouse.

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development. We assessed exercise capacity, muscle morphometry, Cardiac function and ex vivo skeletal muscle fatigue resistance.

Materials and Methods

Ethical approval

This study was approved by the University of California, San Diego, Animal Care and Use Committee and conducted in accordance with the guidelines outlined by the *Guide for the Care and Use of Laboratory Animals.* Mice were housed three to four animals per cage in a vivarium maintained on a 12:12 h day-night cycle and were provided standard chow (Harlan Tekland 8604, Madison, WI) and water ad libitum.

Skeletal myofiber VEGF gene-deleted mouse model

Targeting of skeletal myofiber VEGF gene deletion was accomplished by crossing VEGF*LoxP* mice with mice that express *cre* recombinase under the control of a myogenin promoter-MEF2 enhancer (Myo-Cre) construct (Li et al., 2005). The Myo-Cre-MEF2 construct is activated at embryonic day 9.5. VEGF*LoxP* x Myo-Cre mice were backcrossed to obtain mice that were homozygous for the VEGF*LoxP* transgene and heterozygous for the Myo-Cre transgene. Transgenes were detected by PCR amplification of tail DNA using primers specific for VEGF*LoxP* sequence (forward primer 5′-TCCGTACGACGCATTTCTAG-3′, reverse primer 5′-CCTGGCCCTCAAGTACACCTT-3′) and Myo-Cre sequence (forward primer 5′-CTAGAGCCTG-TTTTGCACGTTC-3′, reverse primer 5′-TGCAA-GTTGAATAACCGGAAA-3′). The line was maintained by crossing heterozygous Myo-Cre mice with mice that do not contain the cre gene. All mice were on a homozygous VEGF*LoxP* background.

Exercise capacity

Incremental speed and endurance tests were performed on a treadmill, as previously reported (Olfert et al., 2009). Mice were familiarized on the treadmill by running at 15 cm/sec for 10–15 min several days prior to testing. Maximal speed was determined by running mice at an initial speed of 40 cm/sec and increasing the speed by 5 cm/sec every minute until exhaustion. For the test of endurance mice were ran at a constant speed of 48 cm/sec (60% of WT group average maximal speed) until exhaustion. The criterion for a mouse reaching exhaustion was a period of 10 sec on the shock-grid at the back of the treadmill.

Skeletal muscle morphology

Capillaries and fibers were detected using the Capillary Lead-ATPase method (Rosenblatt et al., 1987) in 10 μm cryosections. Stained entire transverse sections of each muscle were digitally viewed and stored using a Hamamatsu Nanozoomer Slide Scanning System. Total capillary number, total fiber number, and fiber area were measured as previously described (Olfert et al., 2009) and used to calculate the capillary to fiber ratio (C:F), capillary density and mean fiber area.

Transthoracic echocardiography

Animals were anesthetized with 5% isoflurane for one minute and then maintained at 1% throughout the examination. The anterior chest wall was shaved and then Nair was applied to remove any remaining hair. Small needle electrodes for simultaneous electrocardiogram were inserted into one upper and one lower limb.

Transthoracic echocardiography (M-mode, 2-dimensional and Doppler) is performed using the FUJIFILM VisualSonics Inc., Vevo 2100 high-resolution ultrasound system with a linear transducer of 32-55MHz. Measurements of heart rate (HR), left ventricular internal diameter, diastole (LVIDd) and left ventricular internal diameter, systole (LVISDs), interventricular septal thickness, disastole (IVSd) and LV posterior wall thickness, diastole (LVPWd) were determined from the LV M-mode tracing. Percent fractional shortening (%FS) was used as an indicator of systolic cardiac function.

Invasive hemodynamic analysis

A 1.4F micromanometer catheter (Millar Instruments) was inserted retrograde into the aorta via the left carotid artery and advanced into the left ventricle in anesthetized mice (100 mg/kg of ketamine and 10 mg/kg of xylazine, i.p.). Baseline pressure measurements were obtained. Then, graded dobutamine doses of 0.75, 2, 4, 6, and 8 μg/kg/min were administered for 3 min at each dose. Data were reported after bilateral vagotomy. Measurements of LV hemodynamic parameters, including peak LV pressure, LV end-diastolic pressure (EDP), LV dP/dtmax (an index of myocardial contractility), and LV dP/dtmin and tau (time constant of LV relaxation), both indices of relaxation, were recorded as previously reported (Giordano et al., 2001; Olfert et al., 2009; Tang et al., 2013).

In vitro skeletal muscle contractile function

Extensor digitorum longus (EDL) and soleus were removed and electrically stimulated to contract in vitro (EDL: 16V, 300 ms train duration, 0.5 ms pulse duration; soleus: 16V, 500 ms train duration, 0.5 ms pulse duration) in Tyrode's solution, pH 7.4 as previously described (Tang et al., 2010). Fatigue resistance was evaluated by a repetitive contraction protocol: (70 Hz for EDL and 50 Hz for soleus) each 8 sec and then train freq. was increased each minute (for EDL) or every 2 min (for soleus) to 1 contraction each 4, 3, 2, and 1 sec, until the initial force had fallen to 50% (fatigue point).

Statistical analysis

A Student's test was used to determine statistical differences in body weight, muscle mass, morphometric parameters, exercise speed and endurance and muscle fatigue. Differences in VEGF levels in locomotor muscles between genotypes were determined using a 2-way ANOVA and Tukey post-hoc test. A 2-way ANOVA was also used to examine the interaction of genotype and dobutamine on the cardiac parameters. For all analyses $P < 0.05$ was considered significant.

Results

Efficient and selective inhibition of VEGF expression in skeletal muscle

Efficient and specific reduction of VEGF protein levels was limited to skeletal muscles. VEGF levels were decreased in the soleus (64%, $P < 0.0001$), plantaris (94%, $P < 0.0001$), gastrocnemius (75%, $P < 0.0001$) and EDL (99%, $P < 0.0001$) from skmVEGF−/− mice compared to the WT group. No reduction in the level of VEGF expressed by the heart of skmVEGF−/− mice was observed compared to WT mice (Fig. 1).

Exercise limitation

Maximal treadmill speed during the incremental test was reduced by 19% ($P = 0.006$) in skmVEGF−/− mice. Endurance, assessed as the time to fatigue when mice were run on a treadmill at 60% of the WT average maximal speed, was 77% ($P = 0.0004$) lower in skmVEGF−/− mice than the WT group (Fig. 2).

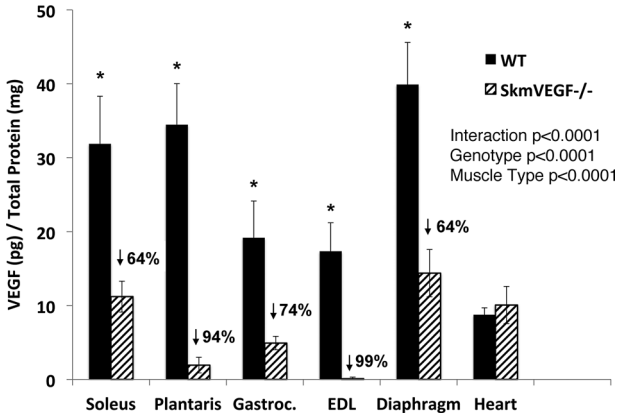

Fig. 1. Skeletal muscle (but not cardiac) VEGF levels are decreased in skeletal myofiber VEGF gene deleted mice (skmVEGF−/−). VEGF levels were measured by ELISA in the soleus, plantaris, gastrocnemius, EDL, diaphragm, and heart from skmVEGF-/ mice and wild-type (WT) littermates. Values are the mean ± SD, n = 3–8. *Indicates a difference between WT and skmVEGF−/− in each muscle type, $P < 0.0001$.

Body weight, muscle mass, and muscle capillary and fiber morphometry

Body weight was 7.4% ($P = 0.02$) lower in skmVEGF−/− mice than WT mice. Individual muscle type mass per body weight was not different between groups (Table 1). Compared to WT mice, a lower capillary to fiber ratio (C:F) was found in the diaphragm (43%, $P = 0.001$), plantaris (23%, $P = 0.01$), gastrocnemius (14%, $P = 0.01$) and EDL (16% lower, $P = 0.03$) of skmVEGF−/− mice. No change in C:F was observed in the soleus. Capillary density was also decreased in the diaphragm from skmVEGF−/− mice. No change in capillary density was observed in the soleus, plantaris, gastrocnemius, or EDL. Fiber cross-sectional area (FCSA) was reduced in the EDL of skmVEGF−/− mice (by 17%, $P = 0.03$). No change in FCSA was detected in the soleus, plantaris and gastrocnemius (Table 2).

TABLE 1. Body weight and muscle mass

	Wild-type	SkmVEGF−/−
Body weight (g)	22.8 ± 1.6	21.1 ± 1.5*
Soleus (mg)/ BW (g)	0.27 ± 0.03	0.33 ± 0.04
Gastrocnemius (mg)/ BW (g)	4.83 ± 0.23	4.77 ± 0.13
TA (mg)/ BW (g)	1.34 ± 0.05	1.46 ± 0.18
EDL (mg)/ BW (g)	0.32 ± 0.03	0.35 ± 0.06
Heart (mg)/ BW (g)	5.37 ± 1.42	4.93 ± 1.76

Values are the mean ± SD.
Body weight (BW), n = 11, Muscles, n = 6.
Age 4–5 months.
*Indicates $P < 0.05$.

Cardiac functional response to dobutamine and echocardiography.

To approximate whether cardiac function contributes to the limitation in whole body exercise, cardiac functional parameters were monitored in anesthetized mice during both a basal state and in response to increasing doses of dobutamine. An increase in dobutamine-responsive heart rate (HR), maximum pressure, Maximum dP/dt, and lower Minimum dP/dt suggest an overall improvement in cardiac function in the skmVEGF−/− mice compared to the WT group.

Post-hoc testing did not reveal significant differences between WT and skmVEGF−/− mice at individual dobutamine doses (Fig. 3). Under basal conditions transthoracic echocardiography did not reveal any differences in heart rate (HR) (WT, 506 bpm ± 36, skmVEGF−/−, 497 bpm ± 49), IVSd (WT, 0.70 mm ± 0.05, skmVEGF−/−, 0.64 mm ± 0.07), LVPWd (WT, 0.70 mm ± 0.03, skmVEGF−/−, 0.65 ± 0.04), LVIDd (WT, 506 mm ± 36, skmVEGF−/−, 497 ± 49), LVIDs (WT, 1.92mm ± 0.48, skmVEGF−/−, 1.83 ± 0.26), fractional shortening (WT, 42.3% ± 5.7, skmVEGF−/−, 41.7% ± 5.8), Mean Vc,f (WT, 9.43 circ s^{-1} ± 1.67, skmVEGF−/−, 9.64 circ s^{-1} ± 0.70) or tau (WT, 14.14 ± 2.08, skmVEGF−/−, 14.87 ± 3.25).

Locomotor contractile function

There was an increase in the time to fatigue (defined as the time at which force fell to 50% of its initial value) in isolated soleus and EDL measured ex vivo - greater by 49 ± 15 sec ($P = 0.0008$) (soleus) and 41 ± 15 seconds ($P = 0.02$) (EDL) in

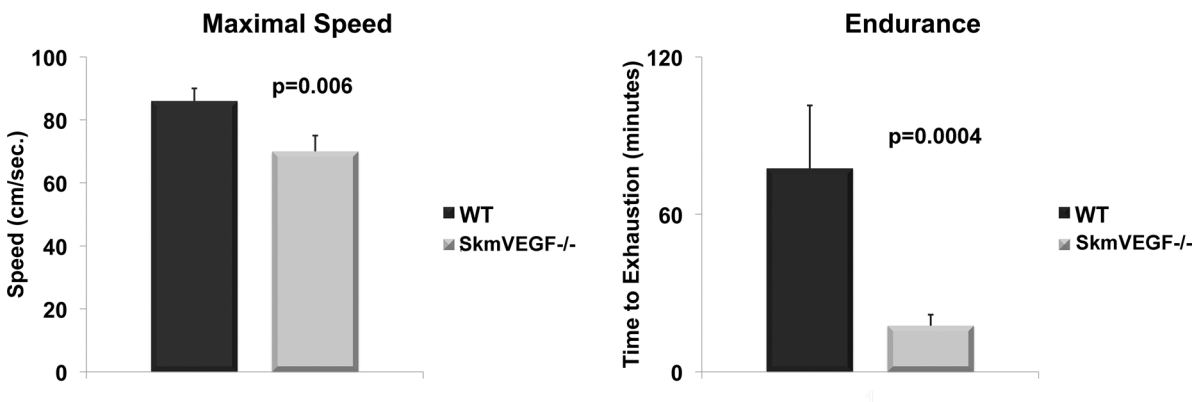

Fig. 2. Decreased exercise capacity in skeletal myofiber VEGF gene deleted mice. At 4 months of age groups of WT and skmVEGF−/− mice were exercise tested on a treadmill. Left: Maximal speed. Right: Endurance. Values are the mean ± SD, n = 6.

TABLE 2. Skeletal muscle capillary and fiber morphometry

	Wild-type	SkmVEGF−/−	*P* value	n
Soleus				
C:F	1.60 ± 0.11	1.46 ± 0.28	0.26	3–5
Density	780 ± 107	733 ± 104	0.35	
FCSA	2067 ± 204	1984 ± 102	0.74	
Plantaris				
C:F	1.51 ± 0.12	1.16 ± 0.20*	0.01	4–5
Density	736 ± 63	669 ± 85	0.23	
FCSA	2063 ± 221	1822 ± 88	0.13	
Gastrocnemius				
C:F	1.61 ± 0.08	1.39 ± 0.08*	0.01	6
Density	591 ± 80	526 ± 60	0.18	
FCSA	2767 ± 277	2680 ± 337	0.66	
EDL				
C:F	1.36 ± 0.07	1.14 ± 0.13*	0.03	5
Density	873 ± 56	871 ± 87	0.93	
FCSA	1586 ± 126	1317 ± 179*	0.03	
Diaphragm				
C:F	1.39 ± 0.18	0.79 ± 0.16*	0.001	5
FCSA	960 ± 135	983 ± 135	0.82	
Density	1301 ± 181	812 ± 103*	0.002	

Values are the mean ± SD.
*Indicates $P < 0.05$.
Capillary to Fiber Ratio (C:F). Capillary Density (Density). Fiber Cross-Sectional Area (FCSA).

muscle from skmVEGF−/− mice than the control group (Fig. 4). No difference in maximal force produced was detected (*data not shown*).

Discussion

These data demonstrate that VEGF expressed by skeletal myofibers themselves (as opposed to VEGF expressed in muscle endothelial cells, satellite cells or possibly fibroblasts and smooth muscle cells) during postnatal development is essential for establishing capillary networks in skeletal muscle of adult cage-confined mice. When VEGF was selectively reduced in skeletal muscle, the most prominent change was observed in the diaphragm. The number of capillaries per diaphragm myofiber measured in skmVEGF was lower than WT by 43%. A similar and severe (~80%) impairment in treadmill endurance exercise capacity, as that reported by Olfert (Olfert et al., 2010) in the combined life-long, cardiac/skeletal myofiber VEGF null mouse, was observed in the present study, in which VEGF gene deletion was targeted exclusively to skeletal myofibers. Interestingly, positive compensations in contractile function and in vitro fatigue resistance were detected, respectively, in both the cardiac and skeletal muscle of life-long, skmVEGF−/− mice. However, these modest improvements in muscle function were not sufficient to maintain normal running speed and endurance levels during treadmill testing.

Comparison of life-long global-, cardiac- and skeletal-myofiber VEGF gene-deleted mice

In whole body exercise tests on a treadmill, which is an integrated outcome of cardiac, respiratory and skeletal muscle function, both the combined cardiac and skeletal myofiber VEGF gene- deleted mice reported by Olfert (Olfert et al., 2009) and the life-long skmVEGF−/− reported here display similar decreases in running speed (global myoVEGF−/− 31% and skmVEGF−/− 18%) and endurance (global myoVEGF−/− 81% and skmVEGF−/− 77%) - Table 3. This suggests that it is VEGF-dependent functions in locomotor and/or respiratory muscles that are essential for achieving normal exercise capacity. This would imply that the reduced cardiac VEGF expression in the global (combined cardiac and skeletal)

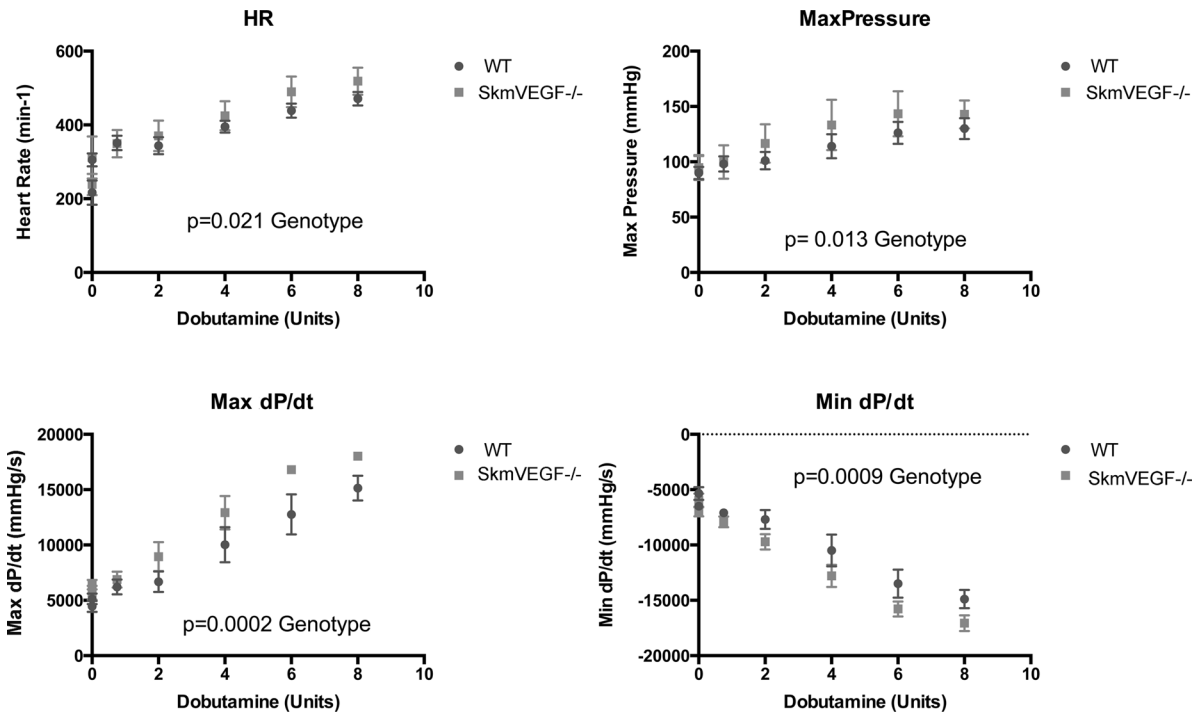

Fig. 3. Cardiac function is enhanced in skmVEGF−/− mice. Hemodynamic measurements of heart rate (HR, min^{-1}), left ventricle Max Pressure (mmHg), Max dP/dt (mmHg/s) and Min dP/Dt (mmHg/s) were made in response to a dobutamine challenge. Values are mean ± SEM, n = 5.

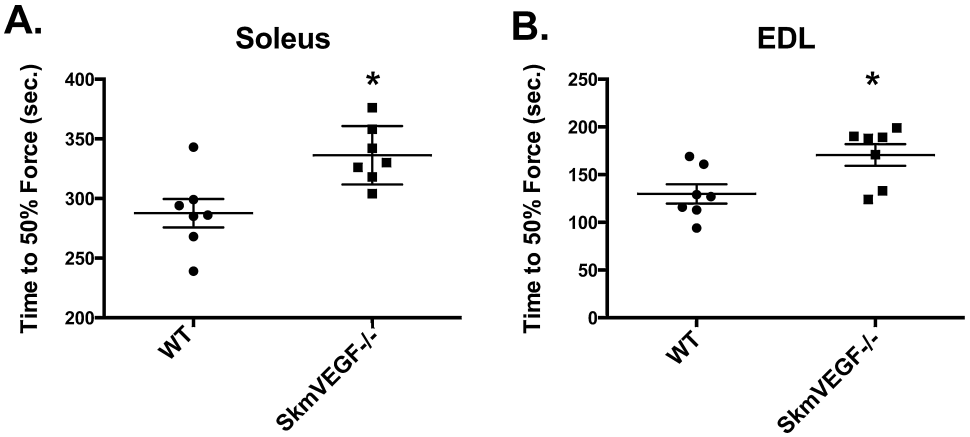

Fig. 4. In vitro skeletal muscle fatigue resistance is improved in skmVEGF−/− mice compared to WT. Time to fatigue (a 50% fall in force produced) was measured in soleus and EDL isolated from WT and skmVEGF−/− mice. Average ± SEM, n = 7, *indicates $P < 0.02$.

myoVEGF−/− mice data reported by Olfert (Olfert et al., 2009) does not play a major role in limiting exercise capacity. However, the cardiac myocyte specific VEGF gene deleted mice reported by Giordano were unfortunately not exercise tested (Giordano et al., 2001) to confirm or refute this conclusion. All three (global-, cardiac- and skeletal-myofiber) VEGF gene deleted mouse lines as adults showed a small decrease in body weight compared to the their wild-type littermates. As expected, the global- myofiber and cardiac myocyte VEGF null mouse lines showed similar impairments in cardiac contractile function. Echocardiographic data collected from skmVEGF−/− mice in the present study did not reveal any differences under basal conditions. Dobutamine challenged life-long skmVEGF−/− mice revealed an overall improvement in functional response when assessed by micromanometer (i.e., heart rate, Max Pressure, Max dP/dt and Min dP/dt). Thus, selectively targeting VEGF gene deletion only in skeletal myofibers revealed a small, yet significant, positive compensation in heart function. This cardiac contractile enhancement, however, was not detectable in the control state but only during testing of contractile reserve by beta-adrenergic receptor stimulation with dobutamine, and was inadequate to protect the mice against a loss of exercise capacity. It remains untested whether cardiac function remained completely normal during exercise in the skmVEGF−/− mice, although this is likely given the exaggerated response to exogenous dobutamine.

In locomotor skeletal muscle, compensatory changes are revealed in both the life-long global- myoVEGF−/− mice (Olfert et al., 2009) and mice with VEGF gene deletion targeted exclusively to skeletal myofibers (skmVEGF−/− mice), present study. In the global myofiber VEGF−/− mice this is evident by increases in metabolic enzyme activities, phosphofructokinase, β-HAD and citrate synthase measured in the gastrocnemius and β-HAD and citrate synthase in the diaphragm. In life-long skmVEGF−/− mice time to fatigue (or fatigue resistance), measured in isolated soleus and EDL, was extended. Thus, skeletal myofiber contractile function is maintained (or even improved) if VEGF is expressed at normal levels in the heart but deficient in skeletal myofibers. These observations would further suggest that it is VEGF-dependent vascular function in the diaphragm and peripheral skeletal muscles that plays a major role in regulating exercise capacity.

A VEGF threshold for attaining a normal number of capillaries per myofiber in peripheral skeletal muscle

Interestingly, the extent of reducing VEGF expression and the resulting outcome in muscle structure differed between locomotor muscle types. In the soleus, which showed the

TABLE 3. Summary of changes in myofiber-targeted VEGF-/- mouse models

	Cardiac myofiber	Cardiac and Skeletal myofiber	Skeletal myofiber	Adult Skeletal myofiber
Target	MLC2v-Cre	Mck-Cre	Myo-Cre-MEF2	HSA-Cre-ER^{T2}
Exercise capacity				
Speed	Not tested	↓ 31%	↓ 18%	=
Endurance	Not tested	↓ 81%	↓ 77%	↓ 30–50%
C:F				
Heart	↓ 40%	↓ 61%	----	----
Diaphragm	----	----	↓ 43%	----
Gastroc.	----	↓ 48%	↓ 14%	=
Cardiac function	↓ Dobutamine Max dP/dt	↓ Dobutamine Max dP/dt	↑ Dobutamine Max dP/dt	----
Compensation	↑ Glut 1 and LDH-A	↑Metabolic enzymes	↑ Fatigue resistance	↑ Metabolic enzymes Fatigue resistance
Study	(Giordano et al., 2001)	(Olfert et al., 2009)	Present study	(Delavar et al., 2014)

smallest decrease in VEGF content (64%), there was no change in capillarity and no difference in the size of the fibers (fiber cross sectional area, FCSA). The gastrocnemius revealed a 74% decrease in the amount of VEGF per total muscle protein that was accompanied by a reduced capillary to fiber ratio (23%) but FCSA was preserved. However, in muscles with dramatic (greater then 90%) decrease in VEGF content, changes in both capillarity and the FCSA were observed. In the EDL a 17% ($P = 0.03$) decrease in FCSA occurred along with a 23% decrease in C:F. In the plantaris, a trend for a reduction in FCSA was observed (12%, $P = 0.13$) and this was in addition to a 16% decreased in C:F. Thus, these data suggest that either VEGF expressed by other cell types within the muscle, or a difference in VEGF gene deletion efficiency between muscle types, allows a functional reserve of VEGF to partially preserve capillaries in some adult skeletal muscle types. In the soleus, which normally has a high capillary density, the endothelial cells may provide this additional source of bioactive VEGF (Lee et al., 2007). Alternatively, muscle types which are actively in use could rely on VEGF from macrophage or progenitor cells recruited to the muscle in response to exercise (Chazaud et al., 2003; Rehman et al., 2003; Rehman et al., 2004; Huntsman et al., 2013), or peripheral nerves that regulate blood flow to active muscle groups (Verheyen et al., 2013). At the present time it is unclear what additional cellular signals or receptor-mediated mechanisms regulate the differential expression in peripheral skeletal myofibers that span an array of oxidative and glycolytic phenotypes.

A major role for VEGF-dependent vascular function in the diaphragm

Interestingly, the two most oxidative muscles analyzed, the diaphragm and soleus, revealed only a 64% decrease in VEGF content as opposed to 74–90% decrease in the other peripheral skeletal muscles analyzed. This would suggest that either 36% of the VEGF expressed is by non-myofiber cell types, with endothelial cells being a likely candidate, or that gene deletion in myofibers is not 100% complete. However, unlike the soleus, which was able to maintain a relatively normal number of capillaries, the diaphragm showed the greatest deficit, with 43% fewer capillaries per fiber compared to a normal WT mice. Together, these data suggest that in diaphragm a decrease in O_2 availability is a factor in limiting exercise capacity. To assess respiratory muscle function in this study, we attempted to obtain arterial blood during exercise and determine if hypercapnia had developed during exercise. Unfortunately, despite repeated attempts we were unable to do so.

Postnatal versus embryonic skeletal myofiber targeted VEGF gene deletion

Several studies have suggested that newly formed capillaries mature and lose their dependence on VEGF in adult organisms (Benjamin et al., 1998; Gerber et al., 1999; Baluk et al., 2004; Kamba et al., 2006). Indeed the early characterization of VEGF*LoxP* mice demonstrated the importance of VEGF on growth and survival of mice was less critical after postnatal week 4 (Gerber et al., 1999). In a later study by Kamba et al. (Kamba et al., 2006) a survey of the VEGF dependence of capillary maintenance in adult organs revealed that several organs (thyroid, adrenal cortex, pituitary, choroid plexus, small-intestinal villi, and epididymal adipose tissue) are dependent on VEGF expression in mice up to 16 weeks of age and this effect was both dose-dependent and variable between organs (Kamba et al., 2006).

Skeletal muscle makes up a large percentage of overall body composition. Furthermore, various skeletal muscles are composed of different combinations of oxidative and glycolytic fiber types with accompanying capillary numbers generally matched to O_2 demand. Our laboratory has previously reported that localized deletion of the VEGF gene in locomotor skeletal muscle, using an AAV vector to deliver *cre* recombinase to VEGF*LoxP* mice, results in substantial capillary regression in adult mice (Tang et al., 2004). The viral vector used in this study was not specifically targeted to myofibers but has the potential to initiate VEGF gene deletion in any cell type residing in the muscle. More recent studies from our laboratory conditionally targeted VEGF gene deletion only in mature myofibers of adult mice and showed that capillary number is not reduced in the soleus, gastrocnemius, plantaris and EDL (Delavar et al., 2014). This is despite very efficient inhibition of VEGF expression in the range of 80–90%. However, VEGF expressed by adult skeletal myofibers appears necessary to form new capillaries in response to exercise training (Delavar et al., 2014).

Summary

Overall this study reveals that reduced life-long locomotor skeletal muscle myofiber- specific VEGF expression contributes substantially and primarily to whole body exercise limitation. A reduction of capillaries supplying mature myofibers, particularly the diaphragm, but also in locomotor muscles, likely limit oxygen available for mitochondrial respiration. While some compensation takes place in both the heart and peripheral skeletal muscle contractility measured in vitro, this is not sufficient to maintain a normal exercise capacity.

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Author Contributions

K.T. designed the experiments and performed the exercise tests and skeletal muscle fatigue measurements. H.W. prepared the histology sections and collected the morphometric data. N.D. performed the cardiac echocardiography. Y.G. measured cardiac hemodynamics paramters. K.L.P. and P.W. supervised studies and provided critical analysis of the data. E.B. designed the study, analyzed the data and prepared the manuscript. All authors contributed to the preparation of the manuscript.

Literature Cited

Baluk P, Lee CG, Link H, Ator E, Haskell A, Elias JA, McDonald DM. 2004. Regulated angiogenesis and vascular regression in mice overexpressing vascular endothelial growth factor in airways. Am J Pathol 165:1071–1085.

Barreiro E, Schols AM, Polkey MI, Galdiz JB, Gosker HR, Swallow EB, Coronell C, Gea J, project EiC. 2008. Cytokine profile in quadriceps muscles of patients with severe COPD. Thorax 63:100–107.

Benjamin LE, Hemo I, Keshet E. 1998. A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. Development 125:1591–1598.

Chazaud B, Sonnet C, Lafuste P, Bassez G, Rimaniol AC, Poron F, Authier FJ, Dreyfus PA, Gherardi RK. 2003. Satellite cells attract monocytes and use macrophages as a support to escape apoptosis and enhance muscle growth. J Cell Biol 163:1133–1143.

Delavar H, Nogueira L, Wagner PD, Hogan MC, Metzger D, Breen EC. 2014. Skeletal myofiber VEGF is essential for the exercise training response in adult mice. Am J Physiol Regul Integr Comp Physiol 306:R86–95.

Gerber HP, Hillan KJ, Ryan AM, Kowalski J, Keller GA, Rangell L, Wright BD, Radtke F, Aguet M, Ferrara N. 1999. VEGF is required for growth and survival in neonatal mice. Development 126:1149–1159.

Giordano FJ, Gerber HP, Williams SP, VanBruggen N, Bunting S, Ruiz-Lozano P, Gu Y, Nath AK, Huang Y, Hickey R, Dalton N, Peterson KL, Ross J, Jr. Chien KR, Ferrara N. 2001. A cardiac myocyte vascular endothelial growth factor paracrine pathway is required to maintain cardiac function. Proc Natl Acad Sci USA 98:5780–5785.

Huntsman HD, Zachwieja N, Zou K, Ripchik P, Valero MC, De Lisio M, Boppart MD. 2013. Mesenchymal stem cells contribute to vascular growth in skeletal muscle in response to eccentric exercise. Am J Physiol Heart Circ Physiol 304:H72–H81.

Kamba T, Tam BY, Hashizume H, Haskell A, Sennino B, Mancuso MR, Norberg SM, O'Brien SM, Davis RB, Gowen LC, Anderson KD, Thurston G, Joho S, Springer ML, Kuo CJ, McDonald DM. 2006. VEGF-dependent plasticity of fenestrated capillaries in the normal adult microvasculature. Am J Physiol Heart Circ Physiol 290:H560–H576.

Lee S, Chen TT, Barber CL, Jordan MC, Murdock J, Desai S, Ferrara N, Nagy A, Roos KP, Iruela-Arispe ML. 2007. Autocrine VEGF signaling is required for vascular homeostasis. Cell 130:691–703.

Li S, Czubryt MP, McAnally J, Bassel-Duby R, Richardson JA, Wiebel FF, Nordheim A, Olson EN. 2005. Requirement for serum response factor for skeletal muscle growth and maturation revealed by tissue-specific gene deletion in mice. Proc Natl Acad Sci USA 102:1082–1087.

Olfert IM, Howlett RA, Tang K, Dalton ND, Gu Y, Peterson KL, Wagner PD, Breen EC. 2009. Muscle-specific VEGF deficiency greatly reduces exercise endurance in mice. J Physiol 587:1755–1767.

Olfert IM, Howlett RA, Wagner PD, Breen EC. 2010. Myocyte vascular endothelial growth factor is required for exercise-induced skeletal muscle angiogenesis. Am J Physiol Regul Integr Comp Physiol 299:R1059–R1067.

Rajagopalan S, Mohler E, 3rd, Lederman RJ, Saucedo J, Mendelsohn FO, Olin J, Blebea J, Goldman C, Trachtenberg JD, Pressler M, Rasmussen H, Annex BH, Hirsch AT, Regional Angiogenesis With Vascular Endothelial Growth Factor t. 2003. Regional angiogenesis with vascular endothelial growth factor (VEGF) in peripheral arterial disease: Design of the RAVE trial. Am Heart J 145:1114–1118.

Rehman J, Li J, Orschell CM, March KL. 2003. Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. Circulation 107:1164–1169.

Rehman J, Li J, Parvathaneni L, Karlsson G, Panchal VR, Temm CJ, Mahenthiran J, March KL. 2004. Exercise acutely increases circulating endothelial progenitor cells and monocyte-/macrophage-derived angiogenic cells. J Am Coll Cardiol 43: 2314–2318.

Rosenblatt JD, Kuzon WM, Jr. Plyley MJ, Pynn BR, McKee NH. 1987. A histochemical method for the simultaneous demonstration of capillaries and fiber type in skeletal muscle. Stain Technol 62:85–92.

Tang K, Breen EC, Gerber HP, Ferrara NM, Wagner PD. 2004. Capillary regression in vascular endothelial growth factor-deficient skeletal muscle. Physiol Genomics 18: 63–69.

Tang K, Murano G, Wagner H, Nogueira L, Wagner PD, Tang A, Dalton ND, Gu Y, Peterson KL, Breen EC. 2013. Impaired exercise capacity and skeletal muscle function in a mouse model of pulmonary inflammation. J Appl Physiol 114:1340–1350.

Tang K, Wagner PD, Breen EC. 2010. TNF-alpha-mediated reduction in PGC-1alpha may impair skeletal muscle function after cigarette smoke exposure. J Cell Physiol 222:320–327.

Verheyen A, Peeraer E, Lambrechts D, Poesen K, Carmeliet P, Shibuya M, Pintelon I, Timmermans JP, Nuydens R, Meert T. 2013. Therapeutic potential of VEGF and VEGF-derived peptide in peripheral neuropathies. Neuroscience 244:77–89.