Skeletal Myofiber VEGF Is Essential for the Vascular Response to Exercise Training in Adult Mice

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science in Biology

by

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2013
The thesis of Hamid Delavar is approved, and it is acceptable
in quality and form for publication on microfilm and electronically:

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Co-Chair

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University of California, San Diego

2013
I dedicate this to my friends and family who have shown unparalleled love and support throughout the years, inspiring me to exceed beyond my goals and strive towards ideals.
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ABSTRACT OF THE THESIS

Skeletal Myofiber VEGF Is Essential for the Vascular Response to Exercise Training in Adult Mice

by

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Master of Science in Biology

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Professor Peter D. Wagner, Chair

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Exercise training stimulates angiogenesis in locomotor muscles. We hypothesize that skeletal myofiber expressed vascular endothelial growth factor is necessary for this angiogenic response to exercise training and overall improvements in exercise capacity. A conditional skeletal myofiber-specific VEGF gene deletion adult mouse model (HSA-Cre-ER\textsuperscript{T2} X VEGF\textit{LoxP}), in which skeletal muscle VEGF levels are reduced by >80%, was used to test this hypothesis. After 8 weeks of exercise training, skmVEGF/- mice were unable to improve maximal speed or endurance. Wild-type littermates showed 18%
and 99% increases in speed and endurance, respectively (p < 0.01). Capillary measurements revealed the angiogenic response (C:F) to training was attenuated in the plantaris of skmVEGF-/- mice (VEGF +/- EX, 38%; skmVEGF/-/- EX, 18%; p < 0.01), and completely inhibited in the gastrocnemius (VEGF +/- EX, 43%, p < 0.01; skmVEGF/-/- 7%, n.s.). Capillarity was unaffected under sedentary conditions. SkmVEGF/-/- mice displayed increased BrdU+ cells (total cell proliferation), vimentin+ cells (fibroblast proliferation) and α-smooth muscle actin (arterial remodeling), which was amplified with training. Contraction-induced blood flow, measured by optical imaging of near-infrared fluorescent nanospheres, was inhibited in skmVEGF/-/- mice. Contractile function of isolated soleus and extensor digitorum longus remained unchanged. SkmVEGF/-/- mice had increased sedentary β-hydroxyacyl-CoA dehydrogenase activity, but training-induced augmentation was completely blocked. Improvements in citrate synthase and phosphofructokinase activity was inhibited and unaltered, respectively. These data suggest VEGF is necessary to augment capillary number and improve exercise capacity in response to exercise training, and plays an essential role in maintaining the vascular system in adult mice.
I:
Introduction
Aerobic exercise training has the ability to modify the vascular system of skeletal muscle by forming new capillaries. This vascular response to exercise is one of many adaptations found in skeletal muscle to more efficiently deliver and utilize oxygen and nutrients by mitochondria in the myofiber. However, the molecular mechanism regulating exercise-induced angiogenesis is not clearly defined. The metabolic hypothesis proposes modification of the vascular system occurs as a consequence of the blood vessel’s inability to provide adequate oxygen supply to meet the increased metabolic demands of tissue (Adair et al., 1990). Studies by Brown and Hudlicka (2003) suggest angiogenesis is likely a response to increased blood flow and accompanying shear stress on vessels located between muscle fibers. While there is likely interconnectedness between these two proposed mechanisms, they ultimate goal is to increase oxygen availability to the muscle though the formation of new capillary networks.

Previous studies have revealed a number of factors involved in capillary formation, and vascular endothelial growth factor-A (VEGF) has proven to be a dominant regulator of angiogenesis (Risau 1997; Carmeliet et al., 2007). VEGF is a member of the platelet-derived growth factor (PDGF) gene family and is expressed as various splice variants (VEGF-A\textsubscript{121}, VEGF-A\textsubscript{165}, and VEGF-A\textsubscript{189}) (Shibuya 2001; Gavin et al. 2004; Gustafsson et al. 2005). VEGF binds to several tyrosine kinase receptors (VEGFR1, VEGFR2, and NRP-1), which dictates its functional response (Neufeld et al., 1999). This exercise responsive gene is transcriptionally upregulated in response to just a single bout of exercise, and thought to be a critical regulator of exercise-induced angiogenesis (Breen et al. 1996; Tang et al., 2009; Olfert et al., 2010). Potential exercise related functions of
VEGF within skeletal muscle include regulation of endothelial cell proliferation involved in forming new vessels, vascular permeability, and neural regulation in peripheral arteries important for exercise-induced hyperemia (Jensen et al., 2004; Lazarus et al., 2011; Jin et al., 2002; Fabel et al., 2003).

Recently, blocking the overexpression of VEGF has been a major focus in antitumor and cancer treatment (Morabito et al., 2006). VEGF signaling, however, is inhibited in a number of chronic diseases such as diabetes, peripheral arterial disease and COPD (Barreiro et al., 2008; Bonner et al., 2013; Wu et al., 2010). These patients are often older individuals and display a number of exercise intolerant symptoms. Such exercise limitations may be contributed to reduced oxygen and nutrient availability to the myofibers, as patients with chronic heart failure (CHF) and chronic obstructive pulmonary disease (COPD) report lower peak oxygen consumption, capillary densities, and capillary-to-fiber ratios in skeletal muscle (Jobin et al. 1998; Duscha et al. 1999). This makes VEGF, and other angiogenic factors linked with improving oxygen metabolism, an attractive field study in the development of potential treatments for these patients.

Several animal models and experiments have been designed to investigate the significance and roles of VEGF and angiogenesis. VEGF plays a critical role in mouse development, as loss of only one allele results in an embryonic lethal phenotype (Carmeliet et al. 1996; Ferrara et al. 1996; Gerber et al. 1999). Tang et al. (2004) have shown that VEGF gene deletion, by delivery of cre recombinase, with a non-cell-specific viral vector, to skeletal muscle of adult VEGFloxP mice, results in capillary regression. Similarly, Giordano et al. (2011) reported decreases in coronary microvessels and thinner
ventricular walls in cardiac myocyte-specific VEGF gene deleted mice. These studies confirm the necessity of VEGF in maintaining capillaries in muscle.

To study the effects of VEGF in response to exercise training in a model of peripheral artery disease, Lloyd et al. (2005) administered a VEGF receptor (VEGF-R) inhibitor to mice with hind limb ligation that were exercise trained. ZD4190 completely blocked the formation of collateral arteries in these mice and only partially inhibited angiogenesis in the exercise trained ischemic muscle. Subsequent studies also observed a decrease in capillary density and capillary-to-fiber ratio in a combined skeletal and cardiac muscle-specific (McK-Cre) VEGF gene ablation mouse model, in which the Mck promoter is first expressed during late embryogenesis. These knockout mice performed poorly when exercise tested compared to wild type mice (Olfert et al. 2009), and were unable to form new skeletal muscle capillaries in the gastrocnemius or improve overall exercise capacity with exercise training (treadmill running) (Olfert et al. 2010).

While these studies suggest that the VEGF gene is necessary to achieve full exercise capacity and capillary regulation in response to aerobic exercise training, they do not address whether skeletal or cardiac muscle specific VEGF is limiting the normal exercise and angiogenic responses in adult mice, or a consequence of the absence of VEGF during development. In skeletal muscle, VEGF is expressed by several cells types [endothelial cells (Lee et al., 2007) smooth muscle cells, fibroblasts (Abou-Khalil et al., 2010) and satellite cells (Christov et al., 2007)], but the majority (80-90%) of VEGF is expressed by myofibers. No current study has addressed the role of skeletal myofiber-specific VEGF in a fully developed mouse with normal vasculature, specifically in response to progressive exercise training.
Therefore, we hypothesized the VEGF expressed by mature skeletal myofibers is essential in regulating the adaptations to exercise training in adult mice. Specifically, we predicted myofiber expressed VEGF in the skeletal muscle is necessary for the normal angiogenic adaptations that contribute to improved exercise capacity with progressive exercise training.

To study the effects of skeletal myofiber expressed VEGF, we used a skeletal myofiber-specific VEGF knockout mouse. Mice with VEGF floxed alleles were developed by placing loxP sites around exon 3 of the VEGF gene, which is present in all isoforms of VEGF-A (Gerber et al. 1999; Robinson & Stringer, 2001; Ferrara et al. 2003). These VEGFloxP transgenic mice were bred with HSA-Cre-ER\textsuperscript{T2} mice (Schuler et al. 2005), in which tamoxifen dependent cre recombinase (Cre-ER\textsuperscript{T2}) is expressed only in skeletal myofibers under the control of human skeletal muscle \(\alpha\)-actin (HSA) sequence. This allows us to conditionally target gene deletion in the myofiber of adult skmVEGF-/- mice that have a fully developed vasculature system. This mouse model more closely models conditions that might occur in those suffering from chronic diseases as adults. We predict that a reduction of VEGF within skeletal myofibers will limit the adaptive response to exercise training, including the capability to increase the number of skeletal muscle capillaries and improve overall exercise capacity.

Chapter 1, in part is currently being prepared for submission for publication of the material. Delavar, Hamid; Nogueira, Leonardo; Knapp, Amy; Wagner, Peter D.; Chambon, Pierre; Metzger, Daniel; Breen, Ellen C. The thesis author was the primary investigator and author of this material.
II: Materials and Methods
Experimental Animals

A colony of mice that have a homozygous VEGFloxP transgene (Gerber et al., 1999) and are heterozygous for HSA-Cre-ERT² (Shuler et al., 2005) 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, USA) and water ad libitum. This study was approved by the University of California, San Diego, Animal Care and Use Committee. All experiments were conducted in accordance to guidelines outlined by the Guide for the Care and Use of Laboratory Animals (National Research Council).

Genotyping

The presence or absence of the HSA-Cre-ERT² transgene in each mouse was determined by PCR analysis using tail DNA (DNeasy Tissue Kit, Qiagen Inc., Valencia, CA). The PCR reaction was run with TaqPro Red Complete DNA Polymerase Master Mix (Denville Scientific, Inc., Metuchen, NJ) and Forward 5’-CTAGAGCCTGTTTTGCACGTTC-3’ and reverse primers 5’-TGCAAGTTGAATAACCGAAA-3’ under the following conditions: one 2-minute polymerase activation incubation at 95°C, 35 cycles of 30 sec. denaturation at 94°C, 30 sec. annealing at 52.1°C, 60 sec. elongation at 72°C, followed by one 8-minute elongation period at 72°C. Reaction products were detected by ethidium bromide staining after agarose gel electrophoresis (OmniPur agarose, EMD Chemicals Inc., Gibbstown, NJ).
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 month old) pre-skmVEGF/- and VEGF +/- mice were weighed and treadmill exercise tested for maximal speed and endurance on two consecutive days. All mice then received tamoxifen from day 0-4 (1 mg/day for 5 days, i.p.). On day 21, post-tamoxifen mice were re-tested for maximal treadmill speed and endurance. SkmVEGF/- EX and VEGF+/- EX mice were exercise trained for 8 weeks while skmVEGF/- UN and VEGF+/- UN mice remained cage-confined. Maximal speed and endurance were re-tested upon completion of this 8-week 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

24-72 hours before exercise testing, mice were familiarized on the treadmill (Model CL-4, Omnitech, Columbus, OH) by running for 10 minutes at 10-15 cm/sec on a 10-degree 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 (60% 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 ($\leq$ 0.3 milliamps) for 10 seconds. Exercise training
consisted of 1 hour of treadmill running 5 days per week, increasing speed from 28 cm/s to 34 cm/s over the course of 8 weeks. All mice received the same treadmill stimulus (seed and duration) throughout 8 weeks of exercise training.

**VEGF Protein Levels**

VEGF protein levels were measured in tissue homogenates using a mouse enzyme-linked immunoassay kit mouse (VEGF Mouse ELISA, R&D Systems, La Jolla, CA). Values were normalized to total protein levels (Bio-Rad DC protein assay).

**Blood Flow Measurements**

Using NIR-fluorescent nanospheres (Qtracker® 800 Non-Targeted Quantum Dots, Molecular Probes, Eugene, OR) and optical imaging (IVIS Spectrum In Vivo Imaging System), blood flow was measured in electrically stimulated (ES) and resting gastrocnemius muscle *in situ*. 40µl of microspheres mixed with 160µl of saline were injected into the mouse via tail vein catheter at a constant rate over the course of 2 minutes. The sciatic nerve of the ES muscle was connected to an electrode and the rest muscle sciatic nerve was severed. Upon injection of spheres, simultaneous images of both electrically stimulated and resting gastrocnemius muscles were collected in the IVIS every 5 seconds (emission: 800nm, excitation: 405-760nm). ES muscle of the mouse was stimulated (0.25 pps stim. rate, 6 V) for 3 minutes total, beginning 30 sec. after injection of microspheres. 80 images were collected during this stimulation periods and for 3 minutes post-ES and analyzed using LiveImaging software.
Skeletal Muscle Morphology

24 hours after the last running sessions, mice were anesthetized (40 mg/kg Ketamine: 3mg/kg Xylazine) and the gastrocnemius complex from one leg was surgically removed, frozen with OCT compound (TBS Tissue Freezing Medium™, Triangle Biomedical Sciences, Durham, NC) in liquid nitrogen-cooled isopentane, and stored at -80°C for sectioning. Capillaries and fibers in 10μm cryosections 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. Total capillary number, total fiber number, fiber areas and type were calculated using ImageJ software.

Ex-vivo Muscle Contractile Function and Fatigue Resistance.

Extensor digitorum longus (EDL) and soleus muscles were carefully dissected from the left hindlimb, mounted in experimental chambers (800MS, Danish Myo Technology, Aarhus, Denmark), and perfused with Tyrode’s solution (in mM: 121 NaCl, 5 KCl, 0.4 NaH₂PO₄, 1.8 CaCl₂, 0.5 MgCl₂, 24 NaHCO₃, 5.5 glucose, 0.1 EGTA) continuously bubbled with 95% O₂ and 5% CO₂ (pH 7.4, 22ºC). Muscles were electrically stimulated (S88X stimulator, Grass Technologies) using square wave pulses (16V; EDL: 300 ms train duration, 0.5 ms pulse duration; soleus: 500 ms train duration, 0.5 ms pulse duration) with single twitches in order to obtain the length that developed maximal twitch tension (L₀). After 15 min resting, “unfatigued” contractile function was evaluated by stimulating the muscles at different frequencies of stimulation (i.e., force-frequency protocol; EDL muscle: 1-150 Hz; Soleus: 1-120 Hz) with 100 sec intervals between contractions. After the force-frequency protocol, muscles rested for 10 min
followed by a fatiguing contractile protocol in order to determine the muscle fatigue resistance. The fatiguing contractile protocol consisted of 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 seconds) each minute (for EDL) or every two minutes (for soleus), until the initial force decreased to 50% (fatigue point). After the experimental procedure, the muscles were blotted dry, weighed, and tension development (in mN) was normalized with respect to the muscle cross-sectional area (mN/mm$^2$). The cross sectional area (in mm$^2$) was calculated dividing muscle mass (in mg) by the product of $L_0$ (in mm) times the density of the muscle (1.06 mg/mm$^3$) (Nogueira et al., 2011).

**Metabolic Enzyme Analysis**

Muscle preparation was performed according to Tang et al. (2010). Briefly, the soleus, plantaris, EDL and one-half of the gastrocnemius muscle (including both medial and lateral portions) were homogenized in (50ul/mg) Passive Lysis Buffer (Promega, Madison, Wisconsin, USA), centrifuged at 10,000g for 10 minutes at 4°C. Supernatant was collected and used to measure activity of citrate synthase (CS), $\beta$-Hydroxyacyl-CoA dehydrogenase ($\beta$-HAD), and phosphofructokinase (PFK). Citrate synthase activity was measured with Citrate Synthase Assay Kit (Sigma, St. Louis, MO), following the manufacture’s protocol (Srere, 1969). $\beta$-HAD activity was measured by the NADH method (Bergmeyer, 1974). PFK activity was measured by the Lowry method (Lowry et al., 1978). Measurements were recorded by spectrophotometer (Beckman DU-640)
readings at room temperature, and enzyme activities are expressed in units (U) of catalyzing mmol substrate/mg tissue/min.

**Immunohistochemistry**

5’-Bromodeoxycytidine (BrdU) was injected (50 mg/kg at a concentration of 10 mg/mL in sterile saline, i.p.) daily for 6 consecutive days before animals were sacrificed. 10µm cryosections of gastrocnemius complex muscle were used to detect BrdU+ cells (Life Technologies), vimentin+ cells (Cell Signaling), and, α-smooth muscle actin+ cells (Sigma, St. Louis, MO). Sections were fixed with 4% PFA, washed in PBS (3 times for 5 min.), treated with 2M HCl at 56°C for 30 min. (BrdU only), blocked in 3% BSA in PBS, incubated overnight in mouse anti-BrdU antibody (0.2 mg/mL), rabbit anti-vimentin antibody (1:200), and mouse anti-actin, α-smooth muscle antibody (1:200) in blocking solution at 4°C. Sections were washed (3 times for 5 min.) in PBS, then treated with appropriate Alexa Flour secondary detection antibody (1:1000) (Invitrogen, Carlsbad, CA). Cells were immunohistochemically analyzed for BrdU (cell proliferation), vimentin (fibroblasts), α-smooth muscle actin (arterial remodeling), and stained with DAPI (4’,6-diamidino-2-phenylindole, Invitrogen, Carlsbad, CA) to visualize nuclei. Confocal microscopy was used to visualize images at 40X.

**Statistical analysis.**

A 2-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 4 experimental groups. P<0.05 is considered significant. All data are presented as mean ± SEM.

Chapter 2, in part is currently being prepared for submission for publication of the material. Delavar, Hamid; Nogueira, Leonardo; Knapp, Amy; Wagner, Peter D.; Chambon, Pierre; Metzger, Daniel; Breen, Ellen C. The thesis author was the primary investigator and author of this material.
III: 
Results
Reduced skeletal muscle VEGF levels in myofiber-targeted gene inactivated mice are not restored with exercise training.

VEGF protein levels are decreased in the soleus, plantaris, gastrocnemius, and EDL muscles by 80, 86, 85, and 76% (p < 0.01), respectively, in untrained skmVEGF-/- mice compared to wild type, untrained VEGF+/+ mice. After 8 weeks of progressive treadmill training, VEGF protein levels increased in the soleus, plantaris, gastrocnemius, and EDL muscles by 23, 21, 19, and 25% (p < 0.01), respectively, in VEGF+/+ EX mice compared to VEGF+/+ UN mice. No differences in VEGF protein levels were observed in the soleus, plantaris, gastrocnemius, or EDL muscles in skmVEGF-/- mice after 8 weeks of exercise training (Figure 1).

SkmVEGF -/- mice do not improve exercise capacity in response to treadmill exercise training.

Maximal treadmill running speed was unchanged in both VEGF+/+ and skmVEGF-/- mice 21 days after administration of tamoxifen to induced VEGF gene deletion. Maximal treadmill speed increased by 18% in VEGF+/+ mice after 8 weeks of exercise training (VEGF+/+ UN, 60.11 ± 1.5 cm/s; VEGF+/+ EX, 69.8 ± 1.1 cm/s; p < 0.01), while no difference in skmVEGF-/- mice was observed (skmVEGF-/- UN, 57.4 ± 1.1 cm/s; skmVEGF-/- EX, 56.2 ± 4.4 cm/s) (Figure 1A). On day 22 post-tamoxifen, skmVEGF-/- mice exhibited a 30% reduction in the time to exhaustion on the treadmill compared to untrained VEGF+/+ mice (skmVEGF-/-, 60.2 ± 6.1 min; VEGF+/+, 86.1 ± 6.6 min; p < 0.01), which was restored after 8 weeks under resting conditions. However, skmVEGF-/- EX mice were unable improve their endurance capacity with exercise
training. Wild-type VEGF+/+ mice improved their endurance capacity by 99% (time to exhaustion: VEGF+/+ UN, 84.1 ± 7.8 min, VEGF+/+ EX, 167.3 ± 5.1 min, p < 0.01) (Figure 1B).

**Body and Muscle Mass.**

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

**Impaired exercise-induced angiogenic response in mice with VEGF-deficient skeletal myofibers.**

Under this exercise protocol VEGF+/+ EX mice increased capillary to fiber ratio (C:F) in the plantaris (40%) and gastrocnemius (43%). Capillary density in VEGF+/+ mice was increased in the soleus (27%), plantaris (38%) and gastrocnemius (49%) with exercise training. Fiber area was unchanged between untrained and exercise trained VEGF+/+ mice. In the skmVEGF/- mice, the only muscle to demonstrate an exercise training response was the plantaris, which revealed an 18% and 25% increases in C:F and capillary density, respectively. The gastrocnemius mean fiber area was reduced by 18% and accompanied by a 6% increase in capillary density, but the C:F remained unchanged (Table 2).
Arterial remodeling in myofiber deficient VEGF mice.

Total BrdU+ cells/myofiber increased in the gastrocnemius of UN and EX skmVEGF-/- mice, compared to 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. Vimentin+ cells/myofiber increased in both UN and EX skmVEGF-/- mice compared to VEGF+/+ UN and VEGF+/+ EX groups in the gastrocnemius (skmVEGF-/ UN, 51 ± 7%; VEGF+/+ 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). Identification of arteries with α-smooth muscle actin (αSM+) showed an increase in the number of arteries per muscle cross sectional area with exercise training in wild-type VEGF+/+ mice, but also with skeletal muscle targeted VEGF ablation (UN skmVEGF-/-) that was further increased with repeated exercise or training (Figure 3A). Analysis of the distribution of arterial size revealed more arteries with a larger diameter in with exercise training and VEGF gene deletion and an interaction between both exercise training and genotype (Figure 3B).

Limited contraction-induced blood flow to the gastrocnemius complex of skmVEGF-/- mice.

Real time optical imaging of blood flow was monitored during electrical stimulation of the gastrocnemius complex in situ. The relative level of fluorescence, representative of blood flow, was simultaneously measured in the contracting and
contralateral, non-contracting muscle and represented by the ratio ES/REST over time during 3 minutes of stimulated contraction and additional 3 minutes post-ES. The ES/REST ratio at the end of the contraction period was 2.30 in the VEGF+/+ group and 1.15 in skmVEGF-/- mice (Figure 4).

**Training-induced augmentation in oxidative metabolic enzymes activities is inhibited in myofiber VEGF deficient mice.**

Citrate synthase (CS) activity increased 49%, 44%, 54%, and 13% in the soleus, plantaris, gastrocnemius, and EDL muscles, respectively, of VEGF+/+ mice after 8 weeks of exercise training. SkmVEGF-/- mice increased citrate synthase activity by only 21%, 22%, and 28% in the soleus, plantaris, and gastrocnemius muscles, respectively. 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 and was not upregulated in response to training in skmVEGF-/- mice. Activity of the glycolytic enzyme phosphofructokinase (PFK) increased in both VEGF+/+ and skmVEGF-/- mice after 8 weeks of exercise training in the soleus (VEGF+/+, 17%; skmVEGF-/-, 11%), plantaris (VEGF+/+, 21%; skmVEGF-/-, 21%), gastrocnemius (VEGF+/+, 21%; skmVEGF-/-, 19%), and EDL muscles (VEGF+/+, 20%; skmVEGF-/-, 22%) (Figure 5). These changes in oxidative enzyme activities did not correlate with a shift to more oxidative type I or IIa fibers in the soleus, plantaris, or gastrocnemius (Figure 6A-C). However, the EDL muscle showed an increase (p < 0.01) in type IIa fibers, and reduction in IIb fibers, in UN skmVEGF-/- mice compared to control UN VEGF+/+ mice (UN skmVEGF-/-, 11.6 ± 1.2%; UN VEGF+/+, 6.4 ± 0.6%).
8 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%) (Figure 6D).

**Ex vivo muscle contractile function**

Peak force and the force-frequency relationship were not affected by exercise training in the soleus and EDL. Peak force was reduced in the soleus muscle of skmVEGF-/− EX mice, yet this did not change the force-frequency relationship (Figure 8). Time to fatigue during a period of repetitive tetanic tension stimulations was measured in the soleus and EDL. EDL isolated from exercise trained skmVEGF-/− showed and increased time to fatigue, or fatigue resistance, compared to the other 3 groups. Exercise training or VEGF gene deletion did not alter isolated soleus fatigue resistance (Figure 9).

Chapter 3, in part is currently being prepared for submission for publication of the material. Delavar, Hamid; Nogueira, Leonardo; Knapp, Amy; Wagner, Peter D.; Chambon, Pierre; Metzger, Daniel; Breen, Ellen C. The thesis author was the primary investigator and author of this material.
Figure 1. VEGF levels in hind limb muscles from wild type and skeletal myofiber VEGF gene ablated mice. VEGF protein in the (A) soleus, (B) plantaris, (C) gastrocnemius and (D) EDL from VEGF+/+ and skmVEGF/-/- mice that were either exercise trained (EXERCISE TRAINED) or remained cage-confined (UNTRAINED) for 8 weeks. Values are mean ± SEM, 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=9-10).
Figure 1. (Continued).
Figure 2. Treadmill exercise capacity. (A) Maximal treadmill speed and (B) endurance were tested in VEGF+/+ and skmVEGF-/- mice at the following time points: pre-tamoxifen, 21 days post-tamoxifen to conditionally delete the VEGF gene, and 8 weeks after cage confinement or exercise training (day 23 to 78). Values are mean ± SEM, 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=9-10).
Table 1. Body mass and skeletal muscle mass to body mass ratio

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<th>VEGF+/+</th>
<th>skmVEGF/-/</th>
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<tr>
<td></td>
<td>Untrained (n=9)</td>
<td>Exercise Trained (n=10)</td>
</tr>
<tr>
<td>Body Mass (g)</td>
<td>24.4 ± 0.70</td>
<td>21.7 ± 0.27*</td>
</tr>
<tr>
<td>Soleus (mg/g)</td>
<td>0.31 ± 0.01</td>
<td>0.31 ± 0.01</td>
</tr>
<tr>
<td>Plantaris (mg/g)</td>
<td>0.74 ± 0.08</td>
<td>0.77 ± 0.06</td>
</tr>
<tr>
<td>Gastrocnemius (mg/g)</td>
<td>4.08 ± 0.19</td>
<td>4.57 ± 0.23</td>
</tr>
<tr>
<td>EDL (mg/g)</td>
<td>0.32 ± 0.01</td>
<td>0.36 ± 0.02</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. *p indicates a significant difference after exercise training within the same genotype (n=9-10). Extensor digitorum longus (EDL).
Table 2. Skeletal muscle morphometry

<table>
<thead>
<tr>
<th>Capillary/Fiber Ratio</th>
<th>VEGF+/+ (n=9)</th>
<th>skmVEGF-/- (n=10)</th>
<th>VEGF+/+ Exercise Trained (n=9)</th>
<th>skmVEGF-/- Exercise Trained (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Capillary/Fiber Ratio</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soleus</td>
<td>1.64 ± 0.06</td>
<td>1.54 ± 0.04</td>
<td>1.82 ± 0.07</td>
<td>1.69 ± 0.05</td>
</tr>
<tr>
<td>Plantaris</td>
<td>1.15 ± 0.03</td>
<td>1.13 ± 0.03</td>
<td>1.61 ± 0.08*</td>
<td>1.33 ± 0.04*</td>
</tr>
<tr>
<td>Gastroc.</td>
<td>1.14 ± 0.03</td>
<td>1.10 ± 0.02</td>
<td>1.63 ± 0.07</td>
<td>1.18 ± 0.05*</td>
</tr>
<tr>
<td>EDL</td>
<td>1.24 ± 0.03</td>
<td>1.12 ± 0.03</td>
<td>1.30 ± 0.02</td>
<td>1.26 ± 0.06</td>
</tr>
<tr>
<td><strong>Mean Fiber Area (µm²)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soleus</td>
<td>1953 ± 116</td>
<td>1795 ± 63</td>
<td>1677 ± 43</td>
<td>1831 ± 144</td>
</tr>
<tr>
<td>Plantaris</td>
<td>1731 ± 63</td>
<td>1895 ± 42</td>
<td>1751 ± 88</td>
<td>1792 ± 87</td>
</tr>
<tr>
<td>Gastroc.</td>
<td>2697 ± 78</td>
<td>2677 ± 81</td>
<td>2601 ± 142</td>
<td>2199 ± 84*</td>
</tr>
<tr>
<td>EDL</td>
<td>1912 ± 89</td>
<td>1936 ± 101</td>
<td>1845 ± 125</td>
<td>2088 ± 162</td>
</tr>
<tr>
<td><strong>Capillary Density (cap/mm²)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soleus</td>
<td>861 ± 59</td>
<td>864 ± 30</td>
<td>1095 ± 48*</td>
<td>968 ± 74</td>
</tr>
<tr>
<td>Plantaris</td>
<td>670 ± 27</td>
<td>599 ± 23</td>
<td>926 ± 42*</td>
<td>751 ± 35*</td>
</tr>
<tr>
<td>Gastroc.</td>
<td>426 ± 12</td>
<td>413 ± 14</td>
<td>635 ± 29*</td>
<td>536 ± 17*</td>
</tr>
<tr>
<td>EDL</td>
<td>661 ± 37</td>
<td>594 ± 38</td>
<td>723 ± 47</td>
<td>629 ± 46</td>
</tr>
</tbody>
</table>

Capillary to fiber ratio, capillary density (capillaries per mm²), and fiber area (µm²) in the soleus, plantaris, gastrocnemius, and EDL muscles of untrained and 8-week exercise trained VEGF+/+ and skmVEGF-/- mice. Values are mean ± SEM, 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.
Figure 3. Arteriogenesis. (A) Total α-smooth muscle actin+ vessels per area (mm²) and (B) histogram of the frequency of arterial diameter size (µm). Gastrocnemius complex cross-sections. Values are mean ± SEM, 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=5).
Figure 4. Morphologic features of skeletal model remodeling. (A) BrdU+ cells, (B) vimentin+ cells, and (C) α-smooth muscle actin+ vessels in the gastrocnemius complex. Images taken by confocal and standard fluorescent microscopy at 40X.
Table 3. BrdU+ cells and Vimentin+ cells, in VEGF+/+ and skmVEGF-/- mice.

<table>
<thead>
<tr>
<th></th>
<th>VEGF+/+</th>
<th>skmVEGF-/-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untrained (n=4)</td>
<td>Exercise Trained (n=4)</td>
</tr>
<tr>
<td>BrdU+ cells/Fiber</td>
<td>0.11 ± 0.02</td>
<td>0.29 ± 0.02*</td>
</tr>
<tr>
<td>Vimentin+ cells/Fiber</td>
<td>0.26 ± 0.04</td>
<td>0.35 ± 0.04</td>
</tr>
<tr>
<td>Vimentin+ Foci/Area</td>
<td>26.6 ± 2.76</td>
<td>37.8 ± 5.88</td>
</tr>
</tbody>
</table>

Values are mean ± SEM, 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.
Figure 5. Contraction-induced blood flow. (A) Optical imaging (IVIS) of NIR fluorescent nanospheres delivered during a period of electrical stimulation (right leg) and rest (left leg) in the gastrocnemius complex of a VEGF+/+ mouse. (B) Relative blood flow during electrical stimulation (30-210 sec.) as a ratio of intensity of fluorescence of ES/REST muscle. Values are mean ± SEM, p < 0.01. Measurements taken in mice 21 days post tamoxifen injection.
Figure 6. Metabolic Enzyme Activity. (A) Citrate synthase (CS), (B) β-hydroxyacyl CoA dehydrogenase (β-HAD), and phosphofructokinase (PFK) activities (µmol/mg/min.) in the soleus, plantaris, gastrocnemius, and EDL. Values are mean ± SEM, 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= 4-9).
Figure 6. (Continued).
Figure 7. VEGF+/+ and skmVEGF/- fiber types. Fiber type percentage for the (A) soleus, (B) plantaris, (C) gastrocnemius, and (D) EDL. Values are mean ± SEM, 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= 9).
Figure 7. (Continued).
**Figure 8. Ex vivo soleus and EDL contractile muscle function.** Contractile function of (A-B) soleus and (C-D) EDL muscles. Muscles were stimulated at the length that developed maximal twitch tension ($L_0$), and normalized to muscle cross-sectional area (mN/mm$^2$) to obtain (A, C) total and (B, D) relative measurements. Values are mean ± SEM, $p < 0.05$. *P indicating significant difference between skmVEGF/-/- EX mice and all groups.
Figure 8. (Continued).
Figure 9. **Ex vivo soleus and EDL fatigue resistance.** Time to fatigue (sec) in the soleus and EDL muscles after a repeated series of tetanic tension stimulations at 50 Hz (soleus) and 70 Hz (EDL). Values are mean ± SEM, \( p < 0.01 \). *P indicates significant difference between skmVEGF/- EX mice and all other groups.
IV:
Discussion
The data collected in this study suggest that VEGF, expressed by skeletal myofibers, is essential in adult mice for improving exercise capacity in response to an 8-week treadmill running program. Contraction-induced blood flow was also decreased due to gene deletion, and the angiogenic adaptation to treadmill running was completely or partially inhibited depending on muscle type. The contractile properties of isolated soleus and EDL muscles were not compromised. Interestingly, there was an increase in the size and number of the arteries in VEGF gene-ablated mice, which was further augmented by exercise training. The training-induced increase in citrate synthase activity was attenuated in skmVEGF-/- mice, yet the glycolytic response to exercise training (PFK enzyme activity), was not altered. The β-HAD response to training was completely inhibited, however, a compensatory increase in β-HAD activity muscle was found in skmVEGF-/- plantaris and gastrocnemius muscles under the sedentary condition. Overall, these data suggest that the vascular response to exercise training is altered in the absence of skeletal myofiber VEGF, and prohibits improvement in running speed and endurance.

A conditional skeletal myofiber-specific VEGF ablation model to inhibit VEGF expression in adult mice.

To approach this study, a mouse model (VEGFloxP X HSA-Cre-ER\textsuperscript{T2}) that allows for the conditional knockout of >80% VEGF within the myofibers of adult mouse skeletal muscle was used. This mouse model was suitable for our study as VEGF gene deletion was solely targeted to skeletal muscle myofibers. Furthermore, these mice are able to develop a normal vasculature system before gene deletion, and this situation more closely models chronic disease and age-related conditions found in older humans.
Decreased VEGF protein levels are observed 21-days post tamoxifen injection (Knapp et al. *unpublished*), and were low at the end of the training period (day 79) in skmVEGF-/- mice. Exercise training of wild-type mice elevated VEGF protein levels, but was not able to restore VEGF levels in skeletal myofiber targeted mice. This is consistent with the study of Waters et al. (2004), which reported increased VEGF levels after 3 and 7 days of voluntary wheel running. The present study now demonstrates the ability of skeletal muscle to uphold this VEGF increase with training over an extended period (8 weeks) of time.

Gute et al. have shown that the intensity of exercise training can have a significant effect on the angiogenic response in skeletal muscle (Gute et al 1994; Gute et al. 1996). Therefore, a training protocol was designed to subject each mouse group to the same absolute exercise intensity. To accomplish this, skmVEGF-/- mice were first run at 60% of their maximal speed (28 cm/s) for one hour each day, and the speed was increased weekly to the highest intensity they could maintain for one hour. Wild-type mice were run at the same speed as the skmVEGF-/- mice. Both exercise and angiogenic responses are clearly evident in VEGF+/+ mice trained. These findings indicate that the protocol used was sufficient to stimulate a training response. Thus, any differences observed between trained mice can be attributed to VEGF gene deletion and not differences between relative training intensities.

**Altered vascular response at rest and in response to exercise training**

These data demonstrate the necessity of myofiber expressed VEGF for the normal adaptations to exercise training. SkmVEGF-/- mice are completely unable to improve
maximal speed or endurance after 8 weeks of training. This limited exercise endurance is present early on at the 21 days post-tamoxifen time point. At the same time there is a significant reduction in contraction-induced blood flow, although the number of capillaries is maintained. Nitric oxide (NO) is a powerful vasodilator and endothelium-relaxing factor (Gruetter et al., 1979; Furchgott & Zawadzki, 1980). VEGF has been shown to activate AMP-activated protein kinase (AMPK), a direct regulator of blood flow, through the endothelial nitric oxide synthase (eNOS) mediated release of NO (Li et al., 2008; Dimmeler et al., 2001). Reduced NO bioavailability, through VEGF-dependent AMPK activation, may contribute to the observed decreased in blood flow. This would result in restricted oxygen availability to the myofiber mitochondria, and limit the endurance capacity in skmVEGF-/- mice.

Capillary number in sedentary skmVEGF-/- mice was unaltered when compared to VEGF+/- untrained mice. This contrasts results found by Olfert et al. (2009), which reported significant fewer gastrocnemius capillaries in a mouse model that inactivated VEGF during late embryogenesis in both cardiac and skeletal myofibers under the control of the Mck promoter. However, the exercise induced angiogenic response in the gastrocnemius is inhibited in both the traditional and conditional VEGF gene deletion mouse models. These data suggest that cardiac and/or skeletal muscle VEGF is important for the development skeletal muscle capillaries. However, in a mature mouse, skeletal myofiber VEGF regulates the angiogenic response to training.

Exercise capacity (time to exhaustion) was restored to normal in skmVEGF-/- mice after 8 weeks of rest. While capillarity remained unchanged in both untrained groups, mice lacking VEGF displayed signs of remodeling, including increased cell and
fibroblast proliferation, and arteriogenesis. This contrasts Lloyd et al.’s (2005) study, which reported a complete block of arteriogenesis when inhibiting VEGF receptor tyrosine kinase activity in mice with hind limb ischemia. Capillary growth in ischemic gastrocnemius muscles was also observed with training in this model of peripheral artery disease, a response that is completely blocked in the current gene ablated mouse model. This inconsistency may be due disparities in VEGF inhibition (cellular source vs. receptor mediated pathway), or mechanistic differences between artery and capillary formation due to pathological ischemia and normal physiologic vascular remodeling in response to exercise training.

**Upregulated arteriogenesis**

Arterial remodeling is a process that increases the diameter of blood vessels in response to an increased blood flow (Heil et al., 2006). While blood flow is decreased 21 days post gene deletion in skmVEGF-/- mice, we have not determined whether 8 additional weeks of rest or exercise training is capable of restoring contraction-induced flow. Exercise is a well-established mechanism to increase collateral blood flow, endothelial wall stress, and expression of growth factors, such as bFGF, which function in a combination of signal cascades to stimulate arteriogenesis (Prior et al. 2004; Yang et al 1995; Van Royen et al. 2001). Without the ability to increase capillarity with training, skmVEGF-/- mice may be unable to provide sufficient oxygen to meet the increased demands of the exercise muscle. Thus, the myofiber may become hypoxic with each exercise bout. This may lead existing vessels to continue to increase arterial diameter, specifically within the adventitia, which is primary composed of fibroblasts and
extracellular matrix (Semenza 2007). Low oxygen tension and increased mechanical forces associated with increased blood pressure, especially in a muscle that may not be able to dilate during exercise, are known signals for initiating arteriogenesis (Prior et al., 2004; Semenza 2007). While increased α-smooth muscle actin+ and vimentin+ cells support the proposed mechanism, we note such a possible adaptation remains unable to improve exercise capacity after 8 weeks of treadmill running in mice lacking myofiber expressed VEGF.

**Muscle type specific adaptations**

Interestingly, we measured different angiogenic responses to training in different muscles types. VEGF gene deletion had no effect on the capillary-to-fiber ratio in the oxidative soleus, as well as the very glycolytic EDL. SkmVEGF-/- mice were unable to form new capillaries in the gastrocnemius in response to exercise training (previously discussed), yet the plantaris, composed mixed fiber types, exhibited an augmented exercise-induced angiogenic response compared to VEGF+/+ EX mice. Lloyd et al. (2005) also reported a partially blocked angiogenic response in exercise-trained mice treated with a VEGF receptor blocker (ZD4190), however this angiogenic response cannot be solely attributed to loss of VEGF in this ischemic mouse model.

While the mechanism of the angiogenic response in the plantaris of skmVEGF-/- mice is unknown, it may stem from a number of factors. VEGF expressed by other cells, such as endothelial cells, satellite cell or macrophage, may be contributing to the observed response. Other angiogenic factors, such as angiopoietin 1 (ANGPT1), which increases blood vessels by recruiting pericytes to the endothelium (Thurston et al. 1999),
may be overexpressed in the absence of skeletal muscle VEGF. Placental growth factor (PGF), platelet-derived growth factor (PDGF), and fibroblast growth factor-2 (FGF2) act in a synergistic fashion when combined with VEGF, and may play a more significant role in this model to compensate for the loss of VEGF (Carmeliet et al. 2001; Luttun et al. 2002; Cao et al. 2003). While any of these mechanisms may account for the angiogenic response in the plantaris of skmVEGF-/− mice, the data demonstrate the necessity of myofiber expressed VEGF to reach a full exercise-induced angiogenic response. Furthermore, this limited angiogenic response resulted in a nonfunctional adaptation, as these mice remained incapable of improving exercise capacity after 8 weeks of progressive training.

**Myofiber compensatory responses to an aberrant vascular function.**

In general, skeletal muscle adapts its oxidative and glycolytic metabolism in response physiological stimuli such as exercise training (Holloszy & Coyle, 1984; Wallberg-Henriksson et al. 1982; Gollnick et al. 1973; Gollnick & Saltin, 1982). Improvements in the glycolytic enzymatic activity of phosphofructokinase (PFK) were unaffected in mice lacking skeletal muscle VEGF following exercise training. Citrate synthase (CS) and β-hydroxyacyl CoA dehydrogenase (β-HAD) activities, markers of oxidative metabolism, were increased in all measured muscles after 8 weeks of exercise training in wild type VEGF+/+ mice. CS and β-HAD activity in skmVEGF-/− mice, however, suggest mitochondrial respiration may be attenuated and lipid oxidation completely blocked after training. However, β-HAD activity in the plantaris and gastrocnemius was upregulated in untrained skmVEGF-/− mice compared to untrained
VEGF\(^{+/+}\) mice. Increased baseline CS, \(\beta\)-HAD, and PFK activity were reported in Olfert’s (2010) results, suggesting a switch in fuel utilization may be a compensation mechanism for the loss of VEGF from birth. Upregulation in oxidative and glycolytic metabolic enzyme activities is also reported in patients of unilateral arterial disease and peripheral artery insufficiency (Jansson et al., 1988; Bylund et al., 1976).

Reduced ATP production due to decreases in oxygen availability may lead to a metabolic adaptation in these patients, as well as in skmVEGF\(^{-/-}\) mice, to maximize energy production through oxidative metabolism. These data support VEGF as critical in reaching the full oxidative and aerobic capacity with exercise training. While skeletal muscle may adapt metabolically to a reduction in VEGF, these adaptations in are unable to improve exercise capacity and offset the lack of angiogenesis.

**Skeletal muscle contractile properties are not impaired in skmVEGF\(^{-/-}\) mice.**

The contractile properties and fiber types of skeletal muscle also adapt in response to exercise (Gute et al. 1994; Hudlicka et al. 1982; Wallberg-Henriksson et al. 1982). Myofiber deficient VEGF mice did not display any major impairment in locomotor muscle contractile function in the soleus or EDL muscles when analyzed in vitro. In fact, fatigue resistance was slightly improved in the EDL of exercise trained skmVEGF\(^{-/-}\) mice. It is unclear why the EDL in the trained KO mice have an improved function. There was a slight shift from type IIb to IIa fibers with training, but this occurred in the wild-type VEGF\(^{+/+}\) group as well. We do note that there are no significant changes in fiber types in the oxidative and mixed fiber type muscles (soleus, plantaris, and gastrocnemius) in VEGF\(^{+/+}\) or skmVEGF\(^{-/-}\) mice with exercise training. Studies on
running wheels, suggest that fiber type transitions in response to training are dependent upon a preceding angiogenic response (Waters et al. 2004). Oueslati and Gardiner (2000) have shown increased EDL fatigue resistance is dependent on the regulation of calcium release by the sarcoplasmic reticulum, and changes in calcium response during maximal tension development and fatigue may play a more significant role in skmVEGF-/- mice during training.

Significance and Summary

This study clearly establishes the critical role of myofiber expressed VEGF for contraction-induced blood flow, the ability to achieve the full angiogenic response to exercise, as well as improvements to exercise capacity with training. While a number of physiological compensations are observed in skmVEGF-/- mice, including increased arteriogenesis, EDL fatigue resistance, and baseline oxidative metabolism, none of these are capable of improving maximal speed or endurance with training. Thus, these data indicate that VEGF within myofibers of skeletal muscle is necessary for 1) the normal adaptations in exercise capacity with training, 2) the full exercise-induced angiogenic response in locomotor muscles, and 3) the ability of vessels and arteries to properly adapt and supply adequate oxygen and nutrient to the myofibers.

Chapter 4, in part is currently being prepared for submission for publication of the material. Delavar, Hamid; Nogueira, Leonardo; Knapp, Amy; Wagner, Peter D.; Chambon, Pierre; Metzger, Daniel; Breen, Ellen C. The thesis author was the primary investigator and author of this material.
V:
References


