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Loss of Adipocyte VEGF Impairs Endurance Exercise Capacity in Mice

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Abstract

Purpose—Reducing vascular endothelial growth factor (VEGF) in adipose tissue alters adipose vascularity and metabolic homeostasis. We hypothesized that this would also affect metabolic responses during exercise-induced stress, and that adipocyte-specific VEGF deficient (adipoVEGF^{-/-}) mice would have impaired endurance capacity.

Methods—Endurance exercise capacity in adipoVEGF^{-/-} (n=10) and littermate control (n=11) mice was evaluated every 4 weeks between 6 & 24 weeks of age using a submaximal endurance run to exhaustion at 20 m/min, 10-degree incline. Maximal running speed, using incremental increases in speed at 30-second intervals, was tested at 25 weeks of age.

Results—White and brown adipose tissue capillarity were reduced by 40% in adipoVEGF^{-/-}, and no difference in skeletal muscle capillarity was observed. Endurance run time to exhaustion was 30% lower in adipoVEGF^{-/-} compared to controls at all time points (p<0.001), but no difference in maximal running speed was observed between the groups. Following exercise (1 hour at 50% maximum running speed), adipoVEGF^{-/-} mice displayed lower circulating insulin, (p<0.001), lower glycerol (p<0.05), and a tendency for lower blood glucose (p=0.06) compared to controls. There was no evidence of altered oxidative damage or changes in carnitine palmitoyltransferase-1 β expression in skeletal muscle of adipoVEGF^{-/-} mice.

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CONFLICT OF INTEREST

None of the authors have any financial or commercial interest in this outcome of this study. The results of the present study do not constitute endorsement by ACSM

Conclusions—These data suggest that VEGF-mediated deficits in adipose tissue blunts the availability of lipid substrates during endurance exercise, both of which likely reduce endurance performance. Surprisingly, we also find an unchanged basal blood glucose, despite lower circulating insulin in *adipoVEGF*^{−/−} mice, suggesting loss of adipocyte VEGF can blunt insulin release and/or increase basal insulin sensitivity.

Keywords

INSULIN; FREE FATTY ACIDS; GLYCEROL; SKELETAL MUSCLE; WHITE ADIPOSE TISSUE

INTRODUCTION

Adipose tissue is highly vascularized, and capillaries that surround adipocytes function to deliver oxygen, nutrients, growth factors, cytokines, stem cells, monocytes and neutrophils via the blood supply. This vasculature also functions to remove waste products from the tissue and to mobilize fatty acids to be used as energy substrates (5). Thus, the vasculature is an important component of adipose development, maintenance and plasticity, which greatly contributes to overall metabolic health. ‘Adipokines’ are released by adipose tissue and are believed to provide vital crosstalk between adipocytes and other organs (e.g. skeletal muscle, pancreas, vascular system, etc.) that is necessary for health, but when dysregulated is associated with metabolic dysfunction and chronic disease (21). However, the direct effect of adipokines leading to metabolic dysfunction and their potential connection to mediators of the vascular system are still poorly understood.

Vascular endothelial growth factor (VEGF) has been classically recognized as a potent, positive regulator of vasculogenesis and angiogenesis (14). The angiogenic actions of VEGF are directed toward endothelial cell functions, but production and secretion of VEGF occurs from a multiplicity of cells in the body, including adipocytes, myocytes, and many others (18, 19). The designation of VEGF as an adipokine is justified by its synthesis in adipose tissue, and recent studies have investigated the specific role of adipocyte-derived VEGF in the context of insulin resistance and adipose dysfunction (8, 25, 26). For example, Elias *et al.* found that mice overexpressing VEGF in adipose tissue were protected against hypoxia and obesity in response to high fat feeding, along with displays of increased insulin sensitivity and glucose tolerance (8). Sun *et al.* have reported similar findings in mice overexpressing adipose VEGF, including transition of white adipose tissue to a more metabolically active “brown-like adipose tissue” phenotype through the potent, local upregulation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) and uncoupling protein 1 (UCP1) (25). Finally, Sung *et al.* have recently show that adipose-specific VEGF deficient (*adipoVEGF*^{−/−}) mice on a high fat diet show lower adipose capillarity, fat pad mass, body mass, and percentage body fat compared to controls (26). *AdipoVEGF*^{−/−} mice also exhibit increased liver steatosis, which often coincides with lipid accumulation in skeletal muscles and induce mitochondrial dysfunction (13, 22). The inability to deposit lipids in adipose tissue could also suggest a concurrent inability to sufficiently mobilize and utilize free fatty acids (FFA) as an energy substrate. Taken together, these observations suggest a potential link between adipose VEGF and alteration of

energy metabolism. While a small number of studies have shown that exercise can increase VEGF mRNA in adipose tissue of mice and rats (1, 10), little is known about the importance of adipose-derived VEGF in context of exercise capacity and metabolism.

The purpose of this study was to evaluate endurance capacity and substrate utilization in response to exercise in *adipoVEGF*^{-/-} mice compared to controls. We hypothesize that *adipoVEGF*^{-/-} mice will have lower endurance running capacity compared to controls, due to reduced ability to mobilize and utilize lipid-derived energy from adipose tissue stores during submaximal exercise. To test this hypothesis, we performed submaximal endurance testing and maximal running speed testing at several time points in *adipoVEGF*^{-/-} and control mice, and subsequently measured levels of circulating glucose, insulin, glycerol and free fatty acids (FFA) in response to a one-hour submaximal run. In addition, basal levels of liver and skeletal muscle glycogen were also measured, as well as protein expression of carnitine palmitoyltransferase-1 β (CPT1 β) in mitochondria isolated from skeletal muscle as an index of lipid transport within the myocyte.

MATERIALS AND METHODS

All experiments were approved by and conducted in accordance with the guidelines of the West Virginia University Institutional Animal Care and Use Committee. All mice were kept on a 12-hr light/dark cycle schedule, housed between 2 and 5 to a cage and given access to a standard chow diet and water *ad libitum*, except when otherwise noted.

Generation of *adipoVEGF*^{-/-} Mice

C57BL/6J mice expressing Cre recombinase (Cre) under control of the fatty acid binding protein 4 (*fabp4*) promoter were obtained from The Jackson Laboratory (stock no. 005069, Bar Harbor, ME). This line was crossed with a C57BL/6 *VEGF-floxed* LoxP line previously obtained from Dr. Napoleone Ferrarra (9) to produce a line in which VEGF expression is “knocked down” in white and brown adipose tissue (*adipoVEGF*^{-/-}). All experimental animals were homozygous for the presence of LoxP sites on the VEGF gene. *AdipoVEGF*^{-/-} mice possessed the *fabp4*/Cre transgene (Cre⁺), while littermate control mice did not express the Cre gene (Cre⁻) (Fig. 1A and 1B).

Genotyping was carried out via 2 separate PCR reactions performed on genomic DNA extracted from tail snips using tail lysis buffer (Allele-in-One, Allele Biotechnology, San Diego, CA). For detection of VEGF LoxP sites, forward primer 5'-TCCGTACGACGCATTTCTAG-3' and reverse primer 5'-CCTGGCCCTCAAGTACACCTT-3' were used. VEGF LoxP PCR products were amplified using 30 cycles of 94°C for 1:15 (min:seconds), 53°C for 1:39, and 72°C for 2:50. For detection of Cre, forward primer 5'-GCATTACCGGTGCGATGCAACGAGTG-3' and reverse primer 5'-GAACGCTAGAGCCTGTTTTGCACGTTC-3' were used. An internal control primer set of forward 5'-ACGTACATGGCTGGGGTGTT-3' and reverse 5'-ACAGTTTACCTGCCCTGAGT-3' were also used in this reaction. Cre PCR products were amplified using 35 cycles of 94°C for 20 seconds, 59°C for 30 seconds, and 72°C for 55 seconds and all products were resolved and visualized using gel electrophoresis.

VEGF Expression in *adipoVEGF*^{-/-} Tissues

Verification of VEGF gene mutation was performed using genomic DNA isolated from adipose tissue (gonadal and subscapular) and gastrocnemius (GA) skeletal muscle via a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). Primer binding sites were situated outside of the LoxP sites, such that a PCR product could only be formed if the region between LoxP sites had been excised by Cre (the alternative product is too large to be amplified). For detection of deletion, forward primer 5'-CTTCATGGACAGGCTTCGGT-3' and reverse primer 5'-GCCCATATTCCAGAGACGGG-3' were used. PCR products were amplified using 30 cycles of 94°C for 30 seconds, 55°C for 45 seconds, and 72°C for 1 min. Products were resolved by electrophoresis on a 1.5% ethidium bromide-stained agarose gel and imaged using Genesnap software (version 7.01) from a digital imager (G-Box Chemi16, Syngene, Cambridge, UK).

To analyze VEGF mRNA, total RNA was extracted from gonadal white adipose tissue (WAT), subscapular brown adipose tissue (BAT) and GA muscle using an RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA). Reverse transcription was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). For detection of VEGF, forward primer 5'-GGCCTCCGAAACCATGAACT-3' and reverse primer 5'-AGCTTCGCTGGTAGACATCC-3' were used. For an internal loading control, *QuantumRNA*TM 18S Internal Standards primers (Ambion®, Austin, TX) were used according to the manufacturer's instructions. PCR products were amplified, resolved and imaged as described above. Relative VEGF expression was determined by normalizing the optical density of the VEGF product band to that of the 18s product band using NIH ImageJ software (version 1.46r).

Exercise Protocols and Body Composition Analysis

AdipoVEGF^{-/-} mice (n=10; 5 males, 5 females) and littermate controls (n=11; 7 males, 4 females) were subjected to sub-maximal endurance exercise testing every 4 weeks, starting at 6 weeks and continuing to 24 weeks of age. Endurance testing was conducted on a rodent treadmill (Columbus Instruments, Exer-6M Treadmill, Columbus, OH) set at a 10° incline after mice had been familiarized to the treadmill. The exercise protocol consisted of warm-up exercise for 5 min at 4 m/min, and then gradual increase in speed (2 min at 10 m/min, 2 min at 15 m/min) up to a set speed of 20 m/min until the animal was unable to continue due to exhaustion. Exhaustion was defined as the point at which the animal could no longer get back on to the treadmill and continue running, despite receiving electric shock from wire grid located at the rear of the treadmill.

At 25 and 37 weeks of age, each animal also completed a maximal running capacity test. This test consisted of running at a 10° incline, beginning with a 5 min warm-up period at 4 m/min and then increasing speed in increments of 2 m/min every 30 sec until exhaustion. Maximal running speed has been shown to correlate closely with oxygen uptake in rodents (11, 29), and is therefore often used as a surrogate for determination of maximal aerobic capacity.

Assessment of circulating metabolites in response to exercise was made following a separate 1-hour sub-maximal exercise bout performed at 20 m/min (10° incline). This speed was used to target exercise intensity between 40–60% of maximal capacity, which is expected to be reliant on fat metabolism. Given the maximal running speed for untrained C57BL/6 mice ranges between 35 and 45 m/min (11, 15, 18), we estimate that our mice performed exercise at an intensity between 44–57% of their maximal effort (with % effort increasing to the higher end of this range with increasing age).

Body composition analysis was conducted on each animal 24 hours prior to the endurance run test at each time point using an EchoMRI small animal body composition analyzer (Model 100H, Houston, TX), which gave *in vivo* measurements of total body fat mass and lean mass.

Oral Glucose Tolerance Test

All blood glucose measurements (TRUEtest, Walgreen Co.) were conducted using a small sample of tail blood. For oral glucose tolerance testing (OGTT), mice were conditioned over 3 days to voluntarily consume a small glucose gelatin pellet as previously described (30). Prior to OGTT, mice were subjected to a 6 hr fast, after which basal blood glucose measurements were taken and each mouse was presented with gelatin containing 3g glucose/kg body mass. Mice were excluded from OGTT analysis if they did not consume the entire piece of gelatin within 2 minutes of presentation. Blood glucose was measured and recorded at 15, 30, 60, 90, and 120 minutes following completion of consumption of the gelatinized glucose bolus.

Measurement of Glucose, Insulin, FFA and Glycerol Response to Submaximal Exercise

Basal 6 hr fasting blood glucose measurements were taken as described above, after which the mice were subjected to 60 min of running at 15 m/min, 10° incline (speed was decreased from 20 m/min used for the periodic endurance run tests in order to account for increased age at this time point). Immediately following running, blood glucose was measured from tail blood with a glucose meter (TRUEtest, Walgreen Co.), and up to 200µl of blood was collected via submandibular puncture for glycerol, FFA and insulin analyses. Serum samples were prepared (clotted 20 mins then spun 2,000g for 10 min at 4°C) and stored at –80°C until further analysis. Insulin was measured via ELISA (Crystal Chem, Inc., Downers Grove, IL) according to the manufacturer's instructions. Glycerol and FFA (Kit #GFA-1, Zen-Bio, Inc., Research Triangle Park, NC) were measured using a plate-based assays according to the manufacturer's instructions. Optical densities for these assays were determined with a microplate reader (BioRad Model 550, Global Medical Instrumentation Inc., Ramsey, MN). Basal fasting serum was collected from each animal as described above several days prior to running and analyzed at the same time as post-exercise serum.

Calculation of the Homeostasis Model Assessment (HOMA) to estimate basal insulin sensitivity was based on the following formula, $HOMA = (\text{insulin uU/mL} \times \text{glucose mg/dL}) / 405$.

Tissue Collection and Processing

At 45 weeks of age, mice were anesthetized with a Ketamine/Xylazine cocktail (Ketaject 100mg/kg, Xylazine 5mg/kg IP) and sacrificed for collection of adipose, skeletal muscle, and various organ tissues. Organs/tissues were excised, weighed, and flash frozen in liquid N₂ unless otherwise noted. Careful attention was made to harvest all of the gonadal WAT and subscapular BAT visible in each animal in order to compare the respective adipose tissue masses, thereafter portions of gonadal WAT and subscapular BAT were fixed in 10% neutral buffered formalin for 72 hours at 4°C in preparation for subsequent histological analysis. The mid-belly from triceps surae muscles from one hindlimb were transversely mounted on a cork base using OCT tissue freezing medium, flash frozen in liquid N₂-cooled isopentane, and stored at -80 °C until processed for morphometry.

To obtain a subsarcolemmal mitochondrial sub-population, quadriceps femoris muscles were excised, weighed, immediately placed in 1.75mL/sample freshly prepared, cold Chappell-Perry buffer (100 mM potassium chloride, 50mM 3-(N-morpholino)propanesulfonic acid, 1mM ethylene glycol tetraacetic acid, 10mM magnesium sulfate, 1mM adenosine triphosphate, pH 7.4) supplemented with a protease inhibitor cocktail (BioVision, Inc. #K271-500), and finely minced with scissors. Each sample was disrupted with a Potter-Elvehjem homogenizer and centrifuged at 800g for 10 min at 4°C a total of 3 times to remove debris. The final supernatant was spun at 10,000g for 10 min at 4°C. The pellet was rinsed, resuspended in Chappell-Perry buffer, spun again at 10,000g for 10 min at 4°C, rinsed with phosphate-buffered saline and resuspended in RIPA buffer (Sigma-Aldrich, St. Louis, MO).

Total protein concentrations were determined using the Lowry method according to the manufacturer's instructions (DC™ Protein Assay, Bio-Rad, Hercules, CA). With the exception of adipose tissue preserved in 10% neutral buffered formalin, all samples were stored at -80°C until further processing.

Histochemistry and Image Analysis

To assess adipose tissue capillarity, fixed WAT and BAT were processed to paraffin blocks, cut into 5µm sections, mounted on glass slides and stained with CD31 antibody for endothelial cell labeling (1:50, ab28364, Abcam, Cambridge, UK). Using light microscopy, up to 10 random non-overlapping images were captured from each sample. For WAT, the capillary-to-adipocyte ratio (C:A) in WAT was determined by dividing the total number of capillaries by the total number of adipocytes in each image. For WAT and BAT, capillary density for each image was determined by dividing the total number of capillaries counted by the area of tissue contained in the field of view. As a surrogate measure of WAT adipocyte size, the number of WAT cells per field of view calculated and reported as WAT cell density (#/mm²). To assess skeletal muscle capillarity, soleus (SOL) and plantaris (PLT) muscles were flash frozen in liquid N₂-cooled isopentane and 8µm transverse cryosections were cut and placed on glass slides. Muscle sections were stained using the alkaline phosphatase and dipeptidylpeptidase capillary staining method (17) and imaged at 20x magnification as described above, with the exception that successive, non-overlapping images were taken to capture the entire area of each muscle. Capillary density was

calculated was calculated number of capillaries per mm², and capillary-to-fiber ratio (C:F) was determined by counting the total number of capillaries and dividing by the total number of muscle fibers in each image.

Protein Analysis

Basal tissue levels of glycogen was assessed using a Glycogen Assay Kit (Abcam #ab65620, Cambridge, MA). Basal protein expression of CPT1 β and protein oxidative damage were analyzed using western blotting. Samples of isolated subsarcolemmal mitochondria in RIPA buffer were passed through a 27G needle 10 times. For CPT1 β , 20 μ g of total protein were reduced, denatured, separated using SDS-PAGE gel electrophoresis, transferred to a nitrocellulose membrane, stained with Ponceau S and imaged. Ponceau S stain was neutralized with 0.1M NaOH and the membrane was rinsed in deionized water and then tris-buffered saline with 0.05% Tween-20 (TBST) before blocking with 5% non-fat dry milk (NFDM) in TBST, and exposed to primary (1:200, H-120: sc-20670, Santa Cruz Biotechnology, Santa Cruz, CA) and secondary (1:1000, anti-rabbit IgG HRP-linked #7074, Cell Signaling Technology, Danvers, MA) antibodies diluted in 1% NFDM in TBST. Chemiluminescent detection was carried out using ECL blotting substrate (Denville Scientific, Inc., South Plainfield, NJ) and images were captured using Genesnap software (version 7.01) from a digital imager (G-Box Chemi16, Syngene, Cambridge, UK). Protein expression was quantified using NIH ImageJ software (version 1.46r) and represented as optical density in arbitrary units (AU), and then normalized to total protein measured from Ponceau S staining.

For analysis of protein oxidative damage the OxyBlotTM Protein Oxidation Detection Kit (EMD Millipore, Billerica, MA) was used according to the manufacturer's instructions.

Statistical Analyses

Statistical analyses were carried out using Statview Software package (v5.0.01, SAS Institute Inc., Cary, NC). Significance was accepted at an α of $p < 0.05$. When comparing adipoVEGF^{-/-} vs. littermate control mice for VEGF expression, tissue capillarity, CPT1 β expression, and oxidative damage, an unpaired student's t test was used. Two-way ANOVA was used on single time point variables (i.e. organ/tissue masses only assessed at sacrifice) to evaluate effect of genotype and sex, whereas repeated measures ANOVA (rANOVA) was used to analyze body mass, percent body fat, endurance running capacity and maximal running speed, glucose tolerance, and response of glucose, insulin, glycerol, and FFA to exercise. We report significant interaction effects, and only performed Student's T-test post hoc testing on significant main effects in the absence of a significant interaction effects. All data are presented as mean $\pm$ standard errors of the mean ($\pm$ SEM).

RESULTS

Tissue VEGF expression and capillarity

Genotyping for Cre and VEGF/LoxP is shown in Figs. 1A–1D. As expected, Cre expression was specific to adipose tissue (Fig. 1C) and we observed a 50% decrease in VEGF mRNA expression in WAT ($p < 0.01$), and a 90% decrease in BAT ($p < 0.01$) in adipoVEGF^{-/-} mice

compared to controls (Fig. 1E). Capillarity density and capillary-to-adipocyte ratio (C:A) was 15% and 40% lower, respectively, in gonadal WAT (?) of *adipoVEGF*^{-/-} compared to controls ($p<0.01$, Fig. 2A and 2C), while capillary density in subscapular BAT was 40% lower in *adipoVEGF*^{-/-} compared to controls ($p<0.05$, Fig. 2A and 2D). Due to the architecture of BAT we not able to reliably measure C:A in BAT. Supporting the specificity of VEGF deletion effect to adipose tissue, we found no difference in hindlimb skeletal muscle capillary density and/or capillary-to-fiber ratio (C:F) between *adipoVEGF*^{-/-} and littermate controls (Fig. 2B, 2E, 2F).

Animal body mass, body fat, and tissue characteristics

Body mass in *adipoVEGF*^{-/-} mice were significantly lower at 24 weeks old compared to controls ($p<0.0001$ for Age $\times$ Genotype Interaction, Fig. 3), however there was significant Genotype $\times$ Sex Interaction ($p<0.05$, Fig. 3). Male *adipoVEGF*^{-/-} mice showed decreased body mass compared to controls at all time-points ($p<0.05$), while there was no difference in body mass according to genotype in female mice (see figure, Supplemental Digital Content 1, Body masses for male and female mice).

Body fat percentage was lower for *adipoVEGF*^{-/-} mice compared controls ($p<0.01$ for Age $\times$ Genotype Interaction, Fig. 3), with a lack of an effect between males and females. The density of gonadal adipocytes was not different between *adipoVEGF*^{-/-} and controls (1244 ± 95 vs 1146 ± 71 #/mm², respectively, $p=n.s.$), indicating average size of adipocytes was similar between *adipoVEGF*^{-/-} and controls.

At the time of sacrifice (45 weeks of age), gonadal WAT mass was 47% lower ($p<0.05$) while subscapular BAT mass was 70% lower ($p<0.001$) in *adipoVEGF*^{-/-} mice compared to controls (Table 1). In general the changes in gonadal WAT and subscapular BAT mass were similar between male and female mice, however males tended to have greater heart masses ($p<0.05$), and in some cases skeletal muscle mass (e.g. gastrocnemius and soleus muscle, $p<0.05$) than their female counterparts (Table 1).

Endurance and maximal running speed performance

Submaximal exercise capacity (i.e. time to exhaustion) was lower by 30% in *adipoVEGF*^{-/-} compared to controls (rANOVA $p<0.001$, Fig. 4A). There was no difference in the age-associated rate of decline between the groups; both groups exhibited a similar 37% decline in time to exhaustion between 6 to 24 weeks of age. There was also no difference in maximal running speed between the groups, and both groups showed the same age-related decline (Fig. 4B).

Blood glucose and circulating metabolites

There were no significant differences in basal blood glucose levels (Fig. 4C and 5A) between *adipoVEGF*^{-/-} and controls (see figure, Supplemental Digital Content 2, Effect of submaximal exercise on circulating glucose and insulin in male and female mice). The basal response to OGTT was also similar between the genotypes, as measured by the 'area under the curve' during OGTT (AUC_{glucose}). Following 1-hour of submaximal exercise, blood

glucose fell in both groups ($p<0.01$), but to a greater extent in *adipoVEGF*^{-/-} mice compared to controls ($p<0.05$, Fig. 5A).

Basal insulin was significantly lower in *adipoVEGF*^{-/-} mice compared to controls ($p<0.05$), and decreased in response to exercise ($p<0.01$) in all mice (Fig. 5B). However, the exercise-mediated decrease in insulin was significantly greater in control compared to *adipoVEGF*^{-/-} mice (Fig. 5B, Time $\times$ Genotype interaction $p<0.05$). Supplemental Fig. 2 shows the separate insulin responses from male and female mice (see figure, Supplemental Digital Content 2, Effect of submaximal exercise on circulating glucose and insulin in male and female mice). Estimation of insulin sensitivity using basal insulin and glucose levels to calculate HOMA revealed significantly lower HOMA values for *adipoVEGF*^{-/-} compared to controls (2.56 ± 0.34 vs 4.53 ± 0.52 , $p<0.01$)

Basal circulating glycerol was not different between *adipoVEGF*^{-/-} and controls, however post-exercise glycerol levels were significantly lower in all *adipoVEGF*^{-/-} mice compared to controls ($p<0.01$, Fig. 5C). Likewise, basal FFA were not different between *adipoVEGF*^{-/-} and controls, but there was a trend for exercise increase FFA in control mice ($p=0.06$, Fig. 5D), that was not seen in *adipoVEGF*^{-/-} mice.

Liver and skeletal muscle metabolite expression

Basal expression of glycogen in liver (6.1 ± 0.4 vs 6.6 ± 0.5 ug/ul) and skeletal muscle (64.7 ± 2.0 vs 72.9 ± 4.7 ug/ul) were not significantly different between *adipoVEGF*^{-/-} and controls, respectively. In addition, there were no significant differences in protein expression of CPT1 β (0.13 ± 0.01 vs 0.16 ± 0.04 AU), nor was there evidence of protein oxidative damage, as measured by protein carbonylation levels (0.77 ± 0.17 vs 0.99 ± 0.11 AU), in subsarcolemmal mitochondria isolated from quadriceps femoris muscles of *adipoVEGF*^{-/-} and control mice, respectively.

DISCUSSION

The principle finding of this study is that adipose-specific VEGF deficient mice, which exhibit up to 40% decline in adipose tissue capillarity, have significantly reduced endurance running capacity without a concurrent decrease in maximal aerobic capacity. While there was no difference in basal tissue (liver and skeletal muscle) glycogen levels based on genotype, *adipoVEGF*^{-/-} mice were found to exhibit lower circulating glucose, insulin, and glycerol compared to littermate controls after an acute 1-hour bout of sub-maximal exercise. Circulating levels of FFA did not increase in *adipoVEGF*^{-/-} mice in response to exercise, suggesting that microvessel rarefaction in adipose tissue can significantly impair endurance exercise capacity by blunting transport of lipid stores needed to fuel exercise.

Basal characteristics of *adipoVEGF*^{-/-} mice

As expected, *adipoVEGF*^{-/-} mice showed lower VEGF mRNA content in WAT and BAT (Fig. 1), and a reduction of adipose tissue capillarity without a significant effect in non-adipose tissue (e.g. skeletal muscle) (Fig. 2). WAT and BAT mass was reduced 2–3 fold in *adipoVEGF*^{-/-} compared to controls (Table 1) along with smaller, but still significant, declines in % body fat (Fig. 3). One explanation for difference in magnitude between

adipose mass and % body fat could be that adipocyte formation was delayed in the *adipoVEGF*^{-/-} mice. The formation of adipose tissue is dependent on progenitor cell differentiation in the stromal vascular fraction of adipose tissue. We did not measure adipocyte turnover or self-renewal, but human studies have shown that less adipocyte turnover can result in metabolic changes related to adipokines, hypoxia and oxidative stress (16). These data are in agreement with the findings of Sung et al. (26), that *adipoVEGF*^{-/-} mice show little change in body mass but a lower body fat percentage and dramatically lower gonadal fat mass under basal conditions. Two additional recent studies show that adipose tissue with deficient VEGF expression are found to be hypoxic, inflammatory and metabolically dysfunctional, especially when challenged with a high fat diet (25, 26), suggesting that adipose tissue capillarity plays a vital role in the health and normal function of adipocytes. It also should be noted that the greater relative decline in adipose mass obtained at sacrifice (at 45 weeks of age) contrasts temporally to our body composition assessments (which were measured between 6–24 weeks of age), and therefore we cannot exclude the possibility that difference in relative magnitude between these variables may be influenced by age.

Given that *adipoVEGF*^{-/-} and control mice exhibited similar basal blood glucose, we were surprised to find that circulating basal expression of insulin was decreased. Unchanged glucose levels in the face of 50% reduction in circulating insulin levels seems to indicate a greater sensitivity to insulin in *adipoVEGF*^{-/-} mice. This idea is supported by a significant decline in the calculated HOMA values in *adipoVEGF*^{-/-} versus controls. At present the mechanism of greater insulin sensitivity is unknown, but these data hint at the possibility that presence of VEGF may have direct influence metabolic pathways in manner that is not fully understood. For example, it is possibility that adipocyte VEGF may play a role in regulating or altering adipokines – which are increasingly shown to exert a central role in insulin resistance, type-2 diabetes and cardiovascular disease (21). There is evidence that *adipoVEGF*^{-/-} mice have reduced adiponectin and increased TNF α expression (26). This raises the possibility that the protective effect of adiponectin on inflammatory-mediated apoptosis on pancreatic β -cells (7, 20, 21) may be lost or reduced in *adipoVEGF*^{-/-} mice, and therefore may explain, at least in part, the reductions in circulating insulin we have observed in our study. It should be noted that this seems to be an adipocyte-specific effect, since circulating basal insulin and glucose have been reported to be unchanged in muscle-specific VEGF deficient mice compared to littermate controls (2). Moreover we found no difference in glycogen levels in either the liver or skeletal muscle. It should also be noted that insulin is a potent up-regulator of VEGF mRNA and a stimulus for VEGF release from adipocytes *in vitro* (16). Future studies are needed to better characterize the metabolic influence of adipose VEGF on insulin homeostasis in this model.

Indeed, it is clear that adipocytes are not just mere storage vesicles, but in fact serve an important endocrine function with crosstalk to multiple organs (i.e. skeletal muscle, pancreas, vasculature, etc.) (21). The fact that *adipoVEGF*^{-/-} mice in this study were able maintain similar blood glucose despite a >40% reduction in circulating insulin compared to controls (without changes in basal liver and muscle glycogen levels), provides additional evidence supporting adipokine crosstalk to other organs, but more importantly suggests that

adipocyte-mediated VEGF signaling plays an important role in influencing metabolic function and peripheral organ insulin sensitivity. It is tempting to speculate that therapeutic interventions aimed at affecting adipose capillarity and/or VEGF expression may be useful in the treating chronic diseases with metabolic dysfunction.

Response to endurance and maximal exercise

AdipoVEGF^{-/-} mice displayed lower endurance running capacity, without a concurrent decrease in maximal running speed (Fig. 4), suggesting an impairment in substrate availability in adipoVEGF^{-/-} mice rather than a change in maximal aerobic capacity *per se*. This supported our finding of lower circulating glucose, insulin, glycerol and (to a lesser extent) FFA in adipoVEGF^{-/-} compared to controls following 1-hour of submaximal exercise (Fig. 5). However, it is worth noting that while both groups experienced the expected response of declining glucose and insulin with exercise, adipoVEGF^{-/-} mice did not exhibit the expected increase in glycerol or FFA in response to exercise (as seen in control mice). We believe that the inability to mobilize FFA during submaximal exercise likely explains, at least in part, the reduced exercise capacity based on a decreased perfusion of the adipose tissue as a result of reduced adipose vascularity. Exercise typically increases blood flow to adipose tissue in order to access and utilize lipid stores (i.e. triglyceride lipolysis) for energy during exercise (3, 4). Therefore, it is likely that the 40% loss of WAT capillarity limited the ability to move FFA into the bloodstream and/or carry FFA away from WAT (6).

It is well accepted that insulin secretion is blunted during prolonged low intensity exercise to help stimulate and promote lipolysis (28). This could also explain the post-exercise deficit in insulin seen in adipoVEGF^{-/-} mice compared to controls. If lipid substrates are not available at an appropriate rate during exercise, this may elicit a feedback loop to further suppress insulin secretion in an attempt to drive lipolysis and increase circulating FFA. Given that under basal conditions, insulin was lower in adipoVEGF^{-/-} mice (Fig. 6) but FFA levels were similar to controls, it could be that reduced insulin helped to increase basal lipolysis and maintain FFA under basal conditions, but that the metabolic demand imposed by exercise was too great for this compensation pathway during exercise. Yet, because basal FFA and glycerol levels in adipoVEGF^{-/-} mice were similar to controls while insulin was significantly lower in adipoVEGF^{-/-} mice, we also can't rule out the possibility that insulin inhibiting influence on lipolysis was increased. Indeed, lowering insulin during exercise would be expected to increase FFA and glycerol in circulation, but this was not observed in adipoVEGF^{-/-} mice. This finding is, in part, supported by evidence showing lower FFA levels following high-fat diet in adipoVEGF^{-/-} mice (26). Regardless of the underlying mechanism, these data hint that VEGF mediated signaling in adipose tissue plays an important role in influencing basic metabolic function during exercise. Future studies will require separate evaluation of the direct effects of VEGF on various cell types versus indirect effects of VEGF via altered angiogenesis in tissues.

Skeletal muscle characteristics of adipoVEGF^{-/-} mice

To rule out the possibility that changes in adipocyte VEGF were leading to concurrent changes in skeletal muscle structure and function that in turn could also influence exercise

performance, we examined skeletal muscle capillarity in plantaris and soleus muscles (Fig. 2), as well as total protein oxidative damage and protein expression of CPT1 β in subsarcolemmal mitochondria from the quadriceps muscle. We found no difference between *adipoVEGF*^{-/-} and littermate controls in any of these skeletal muscle assessments, suggesting that the reduced endurance exercise capacity was not mediated by skeletal muscle deficiencies in capillarity, excessive proteomic oxidative stress, or impair muscle lipid transport via CPT1 β .

Clinical significance

Elevated circulating levels of VEGF have been shown in obese individuals and studies have investigated the prospect of inhibiting VEGF action in adipose tissue as a means of treating obesity (5, 12, 27). However, our findings suggest an important role for adipose tissue capillarity, and/or indirectly VEGF, to maintain basal circulating insulin levels and fatty acid substrate availability during submaximal exercise. These data support the notion that adipose VEGF and capillarity are essential for the maintenance of adipose tissue health and metabolic homeostasis (8, 23, 25, 26). But it is also interesting to note, that the condition of adipose tissue in *adipoVEGF*^{-/-} is under-vascularized, a condition that is observed in human adipose tissue during obesity (24, 26). Given our observation that reductions in adipose capillarity can affect substrate availability during submaximal exercise, prescribing the same exercise regime to lean vs obese subjects may not yield the same physiological benefit or elicit the same cellular responses. Moreover, these data, as well as data from striated muscle-specific VEGF deficient mice (19), highlight concerns for anti-VEGF treatments, (currently most commonly used in cancer therapies) which should take into account the potential effects on adipose and skeletal muscle tissue. By the same token, tissue-specific treatments that can change adipose tissue capillary may also serve as a potential therapeutic interventions for metabolic disorders. Additional studies will be needed before we can determine the full extent of VEGF influence on metabolic homeostasis and whether either VEGF directly or indirectly (through changes in tissue vascularity) influences cellular metabolic function.

In conclusion, we show that adipose VEGF is an essential regulator of adipose tissue capillarity, which can influence metabolic function during prolonged submaximal exercise. In agreement with our hypothesis, a deficiency of VEGF in adipose tissue significantly impairs the availability of free fatty acids during endurance exercise and lowers exercise performance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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and all authors contributed in editing and approving the manuscript. The authors would like to thank Mr. Gavin Washington for his assistance in collecting data.

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Figure 1. DNA and mRNA expression of VEGF in adipose and skeletal muscle tissue of adipoVEGF^{-/-} vs. littermate control mice

PCR products from tail-snip DNA indicating presence (A, lane 1) and absence (A, lane 2) of the Cre transgene at 380 bp, with internal control shown at 109 bp. Homozygosity for presence of VEGF LoxP sites (+/+) indicated by a product at 150 bp (B, lane 1), while homozygosity for absence of these sites (-/-) indicated by a 100 bp product (B, lane 2). Indication of deletion in genomic DNA between LoxP sites shown by 670 bp product in adipoVEGF^{-/-} white adipose tissue (WAT) and brown adipose tissue (BAT) (C, lanes 1 and 3 respectively) and absence of this product in control WAT and BAT (C, lanes 2 and 4 respectively). Absence of this deletion band also observed in both adipoVEGF^{-/-} and control skeletal muscle (C, lanes 5 and 6 respectively). VEGF mRNA expression in adipoVEGF^{-/-} WAT and BAT (D, lanes 1 and 3 respectively) indicated by a 160 bp product. Control tissues include WAT and BAT from control mice (D, lanes 2 and 4 respectively), and adipoVEGF^{-/-} and control skeletal muscle (D, lanes 5 and 6 respectively). 18s rRNA internal standard product shown at 489 bp (D, all lanes). (E) Quantification of relative VEGF mRNA expression in indicated tissues of adipoVEGF^{-/-} vs. control mice (n=4 per tissue, per group). Data presented as mean $\pm$ SEM, **p<0.01 comparing adipoVEGF^{-/-} to control for a given tissue type.

Figure 2. Capillarity density in WAT, BAT, and skeletal muscle of adipoVEGF^{-/-} vs. littermate control mice

(A) Representative images shown at 20x magnification for white adipose tissue (WAT) and 40x for brown adipose tissue (BAT). (B) Representative images shown at 20x magnification for plantaris muscle (PLT). Scale bars represent 100 μ m. (C) Quantification of capillary to adipocyte ratio in WAT (adipoVEGF^{-/-} n=5, control n=4) and (D) capillary density in BAT (adipoVEGF^{-/-} n=2, control n=4). (E) Quantification of capillary to fiber ratio in PLT and (F) soleus muscles (SOL) (adipoVEGF^{-/-} n=9, control n=10). Data presented as mean $\pm$ SEM, * p<0.05

Figure 3. Body mass and percent body fat of adipoVEGF^{-/-} vs. littermate control mice
 Body mass (A–C) and percent body fat (D–F) in adipoVEGF^{-/-} (n=10; male n=5, female 5) and littermate control (n=11; male =7, female=4) mice. Data presented as mean ± SEM. Main effect values reported using repeated measures ANOVA.

Figure 4. Endurance and maximal exercise capacity testing and oral glucose tolerance testing in adipoVEGF^{-/-} vs. littermate control mice

(A) Submaximal endurance capacity and (B) maximal running speed in male and female mice (adipoVEGF^{-/-} $n=10$, control $n=11$). For endurance running, mice ran at 20m/min at a 10° incline until exhaustion. For maximal running speed, mice ran at a 10° incline and running speed was increased by 2m/min every 30 sec until exhaustion. (C) Oral glucose tolerance in 42-wk-old adipoVEGF^{-/-} vs. littermate control mice. Blood glucose over the duration of an oral glucose tolerance test in male and female mice (adipoVEGF^{-/-} $n=10$, control $n=11$). Data presented as mean $\pm$ SEM. Main effect values reported using repeated measures ANOVA.

Figure 5. Submaximal exercise effect on circulating substrates in adipoVEGF^{-/-} vs. littermate control mice

Circulating glucose (A), insulin (B), glycerol (C) and free fatty acids (D) under basal (fasted) and post-exercise conditions in adipoVEGF^{-/-} (n=10) and littermate control (n=11). Exercise consisted of a 1 hour submaximal run at 15 m/min (10° incline), and was precedent with a 6 hour fast. Data as mean $\pm$ SEM. Main effect and interaction values reported using repeated measures ANOVA. ** $p<0.01$, * $p<0.05$ comparing adipoVEGF^{-/-} to control for a given condition. ## $p<0.01$, # $p<0.05$ comparing basal to post-exercise for a given genotype.

Table 1Body and tissue mass at sacrifice (45 week old). Mean $\pm$ SEM.

	Control		AdipoVEGF--	
	n=11 (♂=7, ♀=4)		n=10 (♂=5, ♀=5)	
Body mass, g	♂ 30.9 $\pm$ 1.4 ♀ 26.6 $\pm$ 2.2	29.3 $\pm$ 1.3	♂ 27.0 $\pm$ 1.2 ♀ 24.6 $\pm$ 1.9	25.8 $\pm$ 1.1*
Heart mass, g [†]	♂ 130 $\pm$ 6 [§] ♀ 102 $\pm$ 5	120 $\pm$ 6	♂ 122 $\pm$ 6 [§] ♀ 96 $\pm$ 3	109 $\pm$ 5
Heart/Body mass, mg/g [†]	♂ 4.2 $\pm$ 0.2 ♀ 3.9 $\pm$ 0.2	4.1 $\pm$ 0.1	♂ 4.5 $\pm$ 0.2 ♀ 4.0 $\pm$ 0.2	4.2 $\pm$ 0.2
Spleen mass, mg	♂ 134 $\pm$ 36 ♀ 124 $\pm$ 18	130 $\pm$ 23	♂ 68 $\pm$ 7 ♀ 89 $\pm$ 17	78 $\pm$ 9
Spleen/Body mass, mg/g	♂ 4.2 $\pm$ 0.9 ♀ 4.6 $\pm$ 0.4	4.3 $\pm$ 0.6	♂ 2.5 $\pm$ 0.3 ♀ 3.6 $\pm$ 0.7	3.1 $\pm$ 0.4
Gonadal WAT mass, mg [†]	♂ 1122 $\pm$ 134 ♀ 1059 $\pm$ 303	1099 $\pm$ 130	♂ 582 $\pm$ 78* ♀ 590 $\pm$ 187	586 $\pm$ 104**
Gonadal WAT/Body mass, mg/g [†]	♂ 35.8 $\pm$ 3.3 ♀ 37.8 $\pm$ 8.6	36.5 $\pm$ 3.5	♂ 21.7 $\pm$ 2.2* ♀ 22.5 $\pm$ 5.0	22.2 $\pm$ 2.8**
Subscapular BAT mass, mg [†]	♂ 116 $\pm$ 12 ♀ 90 $\pm$ 18	106 $\pm$ 10	♂ 39 $\pm$ 7** ♀ 26 $\pm$ 7**	33 $\pm$ 5.1**
Subscapular BAT/Body mass, mg/g [†]	♂ 3.8 $\pm$ 0.4 ♀ 3.3 $\pm$ 0.5	3.6 $\pm$ 0.3	♂ 1.4 $\pm$ 0.2** ♀ 1.0 $\pm$ 0.2**	1.2 $\pm$ 0.2**
Gastrocnemius mass, mg [†]	♂ 121 $\pm$ 2 ♀ 107 $\pm$ 8	116 $\pm$ 4	♂ 115 $\pm$ 3 ♀ 101 $\pm$ 6	108 $\pm$ 4
Gastrocnemius/Body mass, mg/g	♂ 3.9 $\pm$ 0.2	4.0 $\pm$ 0.1	♂ 4.3 $\pm$ 0.1	4.2 $\pm$ 0.1

	Control n=11 (♂=7, ♀=4)		AdipoVEGF-/- n=10 (♂=5, ♀=5)	
	♀ 4.1 ± 0.2		♀ 4.1 ± 0.1	
Soleus mass, mg [‡]	♂ 7.2 ± 0.6 [§]		♂ 7.9 ± 1.4	
	♀ 5.0 ± 0.7	6.4 ± 0.6	♀ 5.8 ± 0.5	
Soleus/Body mass, mg/g	♂ 0.2 ± 0.02		♂ 0.3 ± 0.05	
	♀ 0.2 ± 0.02	0.2 ± 0.01	♀ 0.2 ± 0.02	
Plantaris mass, mg	♂ 19.1 ± 1.9		♂ 17.7 ± 2.6	
	♀ 14.9 ± 0.9	17.6 ± 1.4	♀ 15.9 ± 0.5	
Plantaris/Body mass, mg/g	♂ 0.6 ± 0.1		♂ 0.7 ± 0.1	
	♀ 0.6 ± 0.02	0.6 ± 0.06	♀ 0.7 ± 0.03	

WAT, white adipose tissue; BAT, brown adipose tissue; symbol ♂=male and ♀=female;

Two-way ANOVA main effect for genotype ([†]) and sex ([‡]).

* p<0.05 and

** p<0.01 adipoVEGF-/- vs control.

[§] p<0.05 compared to opposite sex in same group.