Impaired Angiogenesis Following Hind-Limb Ischemia in Type 2 Diabetes Mellitus:
Differential Regulation of VEGFR1, R2, and Soluble VEGFR-1

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Abstract:

Objective: Deficient angiogenesis following ischemia may contribute to worse outcomes of peripheral arterial disease (PAD) in patients with diabetes mellitus. Vascular endothelial growth factor (VEGF) and its receptors promote angiogenesis in PAD. We tested the hypothesis that in diabetes excess production of soluble Flt-1 could account for impaired angiogenesis.

Methods: Hindlimb skeletal muscle from diet-induced, type II, diabetic (DM) and age-matched normal chow fed (NC) mice was collected, at baseline and 3 days after hindlimb ischemia, and analyzed for expression of VEGF (n = 10/group), full length and soluble VEGF receptors and downstream VEGF signaling (n = 20/group), using ELISA, RT-PCR and western blots (WB). Results: At baseline, DM mice had increased VEGF (NC vs. DM, 26.6±2.6 vs. 53.5±8.8 pg/mg protein, p<0.05); decreased soluble and membrane bound VEGFR-1 (NC vs. DM; sVEGFR1; 1.44±0.30 vs. 0.85±0.08; VEGFR1 1.03±0.10 vs. 0.72±0.10; WB-densitometry, p<0.05); decreased VEGF signaling (NC vs. DM; p-AKT/AKT 0.76±0.2 vs. 0.38±0.1; p-eNOS/eNOS 0.36±0.06 vs. 0.25±0.04, WB-densitometry; p< 0.05); and no change in VEGF R2. Following ischemia, both DM and NC had comparable increase in VEGF A. Although sVEGFR1 and VEGFR1 expression increased in both groups, the fold-increase from baseline was greater in DM.

Conclusions: In DM, there are higher levels of VEGF, lower VEGFR1 and sVEGFR1 with lower receptor/signaling. Following ischemia, VEGF A increases in both NC and DM but sVEGFR1 and VEGFR1 increases disproportionately in DM, which may limit VEGF ligand binding to VEGFR2 and extent of the adaptive angiogenic response.
Introduction

Angiogenesis is defined as the process of formation of new blood vessels from pre-existing ones, and plays a significant role under both physiological and pathological conditions\(^1\). Vascular endothelial growth factor (VEGF) and its receptor system play a key role in the regulation of angiogenesis\(^2\). VEGF A primarily exerts its downstream cellular effects through two tyrosine kinase receptors, fms-like tyrosine kinase 1 (Flt-1 or VEGF R1) and fetal liver kinase 1 (Flk-1 or VEGF R2)\(^2,3\). In addition, a naturally occurring splice variant of VEGFR1, called soluble VEGFR1 (sFlt-1) binds VEGF with very high affinity and lacks the transmembrane domain, thereby acting as an endogenous inhibitor of angiogenesis by sequestering VEGF\(^4\). sVEGFR1 expression in placenta and its association with ecclampsia has been well documented\(^5,6\). In addition, Ambati et al has recently demonstrated that sVEGFR1 maintains corneal avascularity; thereby providing the first evidence of a physiological role of sVEGFR1 in the cornea\(^7\). Several studies have established the role of VEGF A in neoangiogenesis in the skeletal muscle\(^8-10\); and its potential as a therapeutic agent for patients with peripheral arterial diseases (PAOD) has been under extensive investigation\(^11-13\). However, to date, limited information is available on the expression of soluble VEGFR1 in the skeletal muscle, and its regulation under ischemic or diseased conditions. Understanding the expression of VEGF A and its receptor systems in the skeletal muscle is necessary for proper therapeutic designs.

Diabetes mellitus is characterized by abnormalities in multiple systems. As reviewed by Martin et al, several long-term complications of diabetes mellitus involve abnormalities in angiogenesis, either excessive angiogenesis (retinopathy and nephropathy), or an attenuated angiogenic response implicated in abnormal wound
healing and ulcers. A defective angiogenic response, in part, underlies the increased risk of cardiac diseases in diabetic patients. Mounting evidence indicates that in patients with diabetes, peripheral arterial obstructive disease (PAOD) has worse outcomes compared to non-diabetics. In preclinical models of PAOD, diabetic animals have been shown to have attenuated perfusion recovery in response to induced ischemia.

A relatively reduced angiogenic response in diabetes has been attributed to several factors such as impaired release of endothelial progenitor cells from the bone marrow as well as defective function of progenitor cells. Studies have shown that levels of VEGF A in the plasma as well as in various tissues are higher in diabetics compared to controls. In addition, in pre-clinical and clinical trials, intramuscular delivery of VEGF A has been of limited success in improving measured outcomes. Sasso et al showed that myocardium of patients with type II diabetes have increased expression of VEGFA and a reduced expression of VEGFR1 and R2, in association with a reduction in activation state of downstream signaling molecules. Taken together, as reviewed by Simons et al, blunted angiogenesis in diabetes might be analogous to insulin resistance, i.e. inadequate receptor expression/sensitivity leads to a defective downstream signaling, which in turn leads to an compensatory increase in the ligand. However, another potential mechanism that can explain reduced downstream effects despite an increase in ligand include ligand trapping, thereby decreasing the availability of free, functional ligand. Soluble VEGFR1 is a decoy receptor that binds VEGF A with very high affinity, and has been shown to be an endogenous inhibitor of angiogenesis in other tissues. Therefore, it is possible that while ligand is over expressed as compensation, the available functional ligand is decreased due to an increase in ligand trap. This study was designed
to test the hypothesis that in type II diabetes, an excess production of sVEGFR1 in the skeletal muscle is responsible for the limited availability of the free ligand and hence impaired angiogenesis in response to ischemia.

**Materials and Methods**

*Animal Use.* All animal protocols in this study were approved by Duke University’s Animal Care and Use Committee and conform with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health.

*Mouse Model of Type II Diabetes.* Type II diabetes was induced in mice by feeding a high fat diet (HFD) for 14 weeks \(^{22,24}\). For the diabetic group (DM), 58 male C57BL/6 mice (Jackson Laboratory, Bar Harbour, ME) were started on high fat diet (HFD) at 4 weeks of age. Another 50 male C57BL/6 mice on normal diet served as the controls (NC). After 14 weeks, an intraperitoneal glucose tolerance test (IGTT) was done to determine mice with impaired glucose tolerance. For the IGTT, after overnight fasting with only water ad libitum, a glucose load of 1 mg/kg body weight was given by intraperitoneal injection. Blood glucose was then measured at 30, 60 and 90 min after the glucose load, using a OneTouch Ultra glucometer (LifeScan. Inc, Milpitas,CA). The area under the GTT curve was calculated for both groups. The area under curve of each mouse on HFD was compared to the mean value of the control group, and mice with values above 3 standard deviations of the mean form the control group were used. Eight mice on HFD did not meet the criteria for diabetes and were excluded from the study.
Experiment Design and Groups. A separate group of mice (n = 10 per group) was used for measurement of perfusion recovery after hind limb ischemia. For tissue analysis, both control (NC) and diabetic (DM) mice were divided into two groups, baseline and 3 days after ischemia (n = 40 each group). The tibialis anterior (TA) muscle was harvested from the mice at baseline and 3 days after ischemia for further analysis at protein (n= 20) and mRNA (n = 20) levels.

Surgical induction of hind limb ischemia. Unilateral hind limb ischemia was induced in mice by ligation and excision of the femoral artery as described before. Briefly, mice were anesthetized with a mixture of Ketamine and Xylazine (90 and 10 mg/kg body weight respectively). The left femoral artery was exposed by a paramedian incision and the artery was ligated proximally at origin of the inferior epigastric and distally at the point of bifurcation into popliteal and saphenous arteries. The segment of the artery between the ligations was carefully separated from the femoral vein and excised.

Hemodynamic Assessment. Bilateral hind-limb perfusion was measured using a Laser Doppler Perfusion Imager (LDPI) system (PERIMED, Stockholm, Sweden). Mice were anesthetized with 2% isoflurane and hair was removed from both legs using a depilatory cream. To minimize temperature variations, mice were kept on a heating pad maintained at 37 degrees celcius. At each time point, results were expressed as a ratio of perfusion in the ischemic to the contrlateral normal limb. The immediate post-ligation ratio (Day 0) was set as the baseline and changes in perfusion were calculated as the ratio at the final time minus the baseline ratio.
RNA Measures. Total RNA was isolated and purified using Trizol reagent (Invitrogen, Carlsbad, CA) followed by Qiagen RNA purification kit (Qiagen, Valencia, CA), following manufacturers’ instructions. Total RNA was quantified using a spectrophotometer and 1 microgram of total RNA was reverse transcribed to cDNA using Invitrogen Superscript III Reverse Transcriptase Kit. For reverse transcriptase polymerase chain reaction (RT-PCR), primers sequences and amplification conditions are shown in Table I. For quantitative real-time (qt-RT-PCR), cDNA was quantitatively amplified using TaqMan Universal PCR Master Mix (AB Applied Biosystems, Foster City, CA) and primer-probe sequences as shown in table I. For real-time PCR, initial template concentrations were calculated using the Ct value representing the PCR cycle number at which fluorescence was detectable above an arbitrary threshold, and normalized against endogenous control (18S rRNA). Non-template controls using RNA without reverse transcription were always included, and each sample was tested in triplicate.

Protein Measures. For extraction of total cellular protein, muscle samples were homogenized and centrifuged in Tris-Saline buffer (100mM NaCl, 10mM Tris-HCl, 0.1% Triton and 0.05% Diethylamine and protease inhibitors, pH 7.4). Protein concentrations were determined by De Protein Assay (Bio-Rad, Hercules, CA). VEGF A levels were determined using Quantikine Mouse VEGF A Immunoassay (R&D systems, MN), following manufacturer’s recommendations. Each sample was done in triplicate and the values normalized to total protein. For western blotting, 30-50 micrograms of total protein were separated on SDS-polyacrylamide gels and then transferred to polyvinylidene difluoride membranes. After treating with blocking buffer (5% not fat
milk in PBST) at room temperature for 30 minutes, membranes were incubated overnight at 4°C in the following antibodies: rabbit polyclonal anti Ser473-phospho-Akt (1:500), rabbit polyclonal anti-Akt (1:1000), rabbit polyclonal anti-phospho-eNOS Ser1177 (1:500), rabbit polyclonal anti-eNOS (1:1000) (Cell Signaling, CA), rat monoclonal Anti-VEGFR 2 (1: 500, Silenus, Australia), mouse monoclonal anti-VEGF R1 (1:500) and mouse monoclonal anti-actin (1: 2000) (Sigma-Aldrich, St Louis, MO). Membranes were then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1: 5000, Santa cruz biotechnology, CA) for 1 h at room temperature. Immune reactivity was detected using ECL plus system (Amersham, CA). Results were quantified by densitometry using Image J 1.36b, NIH, USA.

Statistics. All statistical analysis was done using the SPSS software (SPSS 13.0, Chicago, IL). Comparison between the groups was done using independent student’s t-test for baseline values and paired t-test for non-ischemic vs ischemic values. Statistical significance was set at a p value of <0.05. Repeated measures ANOVA was done to assess perfusion improvement over time within groups.

Results

Diabetic mice have impaired perfusion recovery following hind-limb ischemia

After 14 weeks of high fat diet, C57BL/6 mice (n = 50) had significantly greater body weight (43.52 ± 1.8 g vs. 28.2 ± 1.6 g, p<0.05), fasting glucose levels (187.88 ± 8.45 mg/dl vs. 135.79 ± 5.46; p<0.05), and impaired glucose tolerance (area under GTT curve: 29658 ± 1200 vs. 14811 ± 541 mg/dl*min, p<0.05) compared to age-matched normal chow fed mice (n = 50).
Following induction of hind-limb ischemia (n = 10 each group), mice were scanned at d0, d3, d10, d20 and d30 to estimate perfusion recovery. The recovery of perfusion was significantly attenuated in mice with DM compared to NC (Figure 1), at d3 (0.09 ± 0.06 vs. 0.13 ± 0.06, p>0.05) d10 (0.13 ± 0.06 vs. 0.30 ± 0.03, p<0.05), d20 (0.34 ± 0.06 vs. 0.53 ± 0.05, p<0.05) and d30 (0.37 ± 0.04 vs. 0.57 ± 0.03, p<0.001) time points.

**Expression of VEGF A and VEGF signaling at baseline**

Expression of VEGFA was analyzed in TA muscle isolated from diabetic and control mice at baseline. At both mRNA (Figure 2, A; DM = 2.9 ± 0.7 fold of NC, n = 10 per group) and protein levels (Figure 2, B; NC vs. DM 26.6 ± 2.6 vs. 53.5 ± 8.8 pg/mg total protein, n = 10 per group; p<0.05), VEGF A was significantly higher in muscles from the diabetic mice compared to controls. VEGF A in part exerts its downstream effects via the PI3 kinase/AKT pathway, which in turn phosphorylates and activates eNOS. To investigate the downstream effects of VEGF A, we examined the phosphorylation state of AKT and eNOS as indices of VEGF mediated signal transduction. The ratios of pAKT/total AKT (Figure 2, C; NC, 0.76 ± 0.2 vs. DM, 0.38 ± 0.1, as measured by densitometry of western blots; n = 8; p < 0.05) and p-eNOS/total eNOS (Figure 2, D; NC, 0.36 ± 0.06 vs. DM, 0.25 ± 0.04, as measured by densitometry of western blots; n = 8; p< 0.05) were significantly lower in muscle samples from diabetic mice, thereby indicating that despite an increase in ligand, there is a reduced downstream VEGF signaling in diabetic muscles.
Expression of VEGF receptors in NC and DM Muscle

In order to investigate whether reduced VEGF A signaling in the diabetic mice could be attributed to a deficit of receptors, we examined the expression of the predominant angiogenic receptor, VEGF R2. Expression of VEGF R2 was very variable within groups, but there was no significant difference between diabetic and control groups at both mRNA and protein levels (Figure 3, A and B). Interestingly, expression of both membrane bound VEGFR1 (Figure 3, C and D; NC, 1.03 ± 0.10 vs. DM, 0.72 ± 0.10; integrated density VEGFR1/actin; n = 8; p<0.05) and sVEGFR1 (Figure 3, E and F; NC, 1.44 ± 0.30 vs. DM, 0.85 ± 0.08; integrated density sVEGFR1/actin; n = 8; p<0.05) were decreased in muscles from diabetic animals compared to controls.

Expression of VEGF A and VEGF signaling in response to ischemia

Diabetic mice have attenuated perfusion recovery and attenuated neo-angiogenesis after surgical induction of hind limb ischemia compared to NC. To investigate whether there is differential regulation of VEGF system and its signaling in ischemia between diabetic and control mice, we induced unilateral hind limb ischemia in all mice and harvested the TA muscles three days after surgery. In all analysis, expression of proteins in the ischemic limb (I) was compared to that in the contralateral non-ischemic (NI) limb.

In control animals, VEGF A expression in the ischemic (I) muscles significantly increased from that in non-ischemic (NI) muscles (Figure 4, A; NC, NI vs. I 17.30 ± 2.28 vs. 63.79 ± 5.29 pg/mg protein; n = 6 each group; p <0.01). Somewhat unexpectedly, VEGF A was also significantly increased in ischemic muscle from diabetic animals (Figure 4, A; DM NI vs. I 27.83 ± 5.29 vs. 89.19 ± 11.94 pg/mg protein; n = 6 each group; p <0.01). There was no difference in the fold increase from non-ischemic muscle
between the groups (NC 2.6 ± 0.5 vs. DM 3.7 ± 0.9 fold increase from non-ischemic, p > 0.05).

In control mice, VEGF signaling was significantly higher at day 3 after ischemia, as indicated by the ratio of expression of p-AKT to total AKT (NI 0.45 ± 0.01 vs. I 0.51 ± 0.02; ratio of integrated densities as determined from western blots; n = 8; p < 0.05). However, muscles from diabetic mice showed a reduced p-AKT/total AKT ratio at baseline and there was no significant change in the ratio in response to ischemia (NI 0.38 ± 0.03 vs. I 0.36 ± 0.03, determined by densitometry of western blots; n = 8; p > 0.05).

In control mice, both p-eNOS and total eNOS were upregulated following ischemia, and there was no net change in the ratio of p-eNOS/total eNOS (NI 0.109 ± 0.003 vs. I 0.110 ± 0.001, densitometry of western blots; n = 4; p > 0.05). However, in mice with diabetes, the ratio of p-eNOS to total eNOS was significantly lower in the ischemic limb (NI 0.097 ± 0.008 vs. 0.038 ± 0.005; n = 4; p < 0.01).

**Changes in expression of VEGF receptors in response to ischemia**

At mRNA level, no difference in VEGF R2 expression was detected between ischemic and baseline samples in both groups (Figure 5, A). However, at protein level, VEGF R2 expression increased in both groups in response to ischemia, and there was no significant difference between the groups (Figure 5, B; NC, 4.85 ± 1.38 vs. DM, 6.66 ± 0.73 fold increase over non-ischemic muscle, as determined by densitometry from western blots; n = 9). In response to ischemia, the diabetic group had a greater increase in expression of membrane bound VEGFR1 compared to the control group (Figure 5, C and D; NC 2.09 ± 0.32 vs. DM, 6.44 ± 0.76 fold increase from non-ischemic tissue, as determined by densitometry from western blots; n = 9; p < 0.001). Similarly, sVEGFR1 was elevated in
both groups at day 3 after ischemia, indicating a hypoxia inducible transcription of sVEGFR1. This observation was in accordance with findings from other studies in different tissues. However, the magnitude of sVEGFR1 increase in diabetic muscles was much greater than that for the control group (Figure 5, E and F; NC 2.57 ± 0.86 vs. DM, 7.23 ± 2.01 fold increase from non-ischemic tissue, as determined by densitometry from western blots; n = 9; p < 0.05). This indicates that although diabetic muscle is able to increase ligand in response to ischemia, a disproportionate increase in membrane bound and soluble VEGFR1 may limit the availability of sustained levels of free ligand and thereby limit the angiogenic response.

**Discussion**

It is well established both in mice and man that there is impaired angiogenesis in ischemic muscle in diabetes mellitus compared to controls. To the best of our knowledge, findings from this study are the first to describe differential alterations in the VEGF ligand and receptor system as a potential contributor to this problem. The major finds from this study are 1) VEGF A expression was increased in skeletal muscle from diabetic mice compared to that from controls, 2) There was no difference in the expression of VEGF R2 between skeletal muscle from diabetic and control mice, 3) Both membrane bound and soluble VEGFR1 were downregulated in skeletal muscle from diabetic mice, 4) Following ischemia, VEGF A levels increased from baseline in both diabetic and control mice, and 5) Following ischemia, diabetic mice showed a greater increase in expressions of membrane bound and soluble VEGFR1. These finding have implications for advancing our understanding of the VEGF system in health and disease.
Our observation of increased VEGF A expression in skeletal muscles from diabetic mice is in agreement with findings from other investigators in different tissues\textsuperscript{22, 23}. For example, Sasso et al reported an increased expression of VEGF A in the myocardium of diabetic patients with coronary heart disease compared to non-diabetics. This indicates that in diabetics, the ligand may not be the limiting factor. However, whether the excess ligand is the cause or the result of reduced downstream receptor signaling remains to be investigated. Our observation that VEGF mediated signaling is impaired in diabetic mice with elevated VEGF levels and without a significant change in VEGF R2 expression indicates a decreased sensitivity of the receptors to VEGF A in the skeletal muscle.

The role of sVEGFR1 as a ligand trap and as an endogenous inhibitor of angiogenesis has been investigated in other tissues\textsuperscript{4, 6, 7}. The results from our study are the first to show that on sVEGFR1 expression in skeletal muscle have several important implications. First, the observation that sVEGFR1 is present in normal skeletal muscle and is modulated by disease process such as diabetes indicates that sVEGFR1 has important physiological role in the maintenance of baseline angiogenic status. Second, that sVEGFR1 is elevated following ischemia indicates that sVEGFR1 may have a regulatory role in the angiogenic response to ischemia.

Our data also expands information on membrane bound VEGFR1 as it has been shown to have both pro-angiogenic and anti-angiogenic effects based on temporal and tissue dependent manner\textsuperscript{32, 33}. The affinity of VEGFR1 to VEGFA is higher than the affinity of VEGFR 2 to VEGFA, but VEGF R2 has been shown to be the predominant receptor involved in angiogenic process\textsuperscript{3, 33}. Therefore, membrane bound VEGFR1 can
also act as an endogenous inhibitor, by sequestering VEGF A and thereby making it less available for VEGF R2. We found decreased levels of both soluble and membrane bound VEGFR1 in diabetic skeletal muscle at baseline. Collectively, these data suggest that diabetic skeletal muscle undergo several adaptive responses to maintain baseline angiogenic homeostasis, including an upregulation of ligand expression and a downregulation of endogenous inhibitors.

Observation from this study that diabetic muscle can mount an increase in VEGF A in response to ischemia has significant clinical implications. It indicates that proper angiogenic therapeutic targets lie downstream of VEGF in the VEGF signaling cascade. However, despite the indications from these observations, a recent study from our group showed that delivery of ZFP-VEGF, a VEGF activating transcription factor, improves VEGF mediated signaling and perfusion recovery in a mouse model of hindlimb ischemia (ref YJli’s paper). The controversy may be explained by the fact that ZFP-VEGF injection resulted in a sustained induction of VEGF transcription, which was probably sufficient to overcome the sVEGFR1 inhibitory effects and thereby result in a sustained increase in available VEGF.

Our finding that VEGFR2 and VEGFR1 increase in response to ischemia is in concert with observations from other investigators. For example, Li et al have shown that both Flk 1 and Flt1 mRNA are overexpressed in the ischemic zone following coronary artery ligation in a rat model of myocardial ischemia. A noteworthy observation in this study was the discrepancy in expression of VEGF R2 at the level of mRNA and protein at the same time point. While there was no notable change in expression of VEGF R2 mRNA at day 3 after ischemia, there was a 4.8-6.6fold increase at the protein level. This
clearly indicates that measures taken solely at the mRNA level may not be reflective of the expressed protein levels, and thereby have limited functional implications.

Furthermore, our observation of a disproportionate increase in soluble and membrane bound VEGFR1 in diabetic muscle in response to ischemia indicate that modulation of these targets may be an alternative therapeutic option in diabetic patient with PAOD. Jacob et al have shown that delivery of an exogenous form of sVEGFR1 attenuates recovery from hind limb ischemia in a mouse model. Whether inhibition of endogenous sVEGFR1 can improve perfusion outcome remains to be investigated.

In summary, data from this study indicate that diabetic skeletal muscle has increased expression of VEGF A, and comparable expression of VEGF R2, but a reduced downstream signaling, thereby indicating a reduced sensitivity of VEGF R2. In addition, skeletal muscles from diabetic mice show a decreased expression of soluble and membrane bound VEGFR1 at baseline, possibly as an adaptive response to maintain baseline functions. A disproportionate increase in soluble and membrane bound VEGFR1 in diabetic muscle in response to ischemia indicate that sVEGFR1 is part of the pathogenesis of skeletal muscle in diabetes that is associated with a decreased angiogenic response to ischemia, and may thereby prove to be a useful target for novel angiogenic therapeutic approaches in diabetes.
References


Figure Legends

**Figure 1.** Following surgical induction of hind-limb ischemia, perfusion recovery was significantly attenuated in mice with diabetes (DM, n = 10) compared to normal chow-fed control mice (NC, n = 10). The difference was statistically significant from day 10 onwards. Changes in perfusion were calculated by subtracting the immediate post-ligation ratio from the values at the respective time points (*p<0.05 between NC and DM at the specific time points; # p<0.05 compared to previous time point within the group.

**Figure 2.** At baseline, TA muscles from diabetic mice (DM) showed increased expression of VEGF A at both mRNA (A) and protein (B) levels (n = 10; *p<0.05). At baseline, muscles from diabetic mice showed reduced activation state of the downstream second messengers for VEGF, as indicated by reduced phospho-AKT/total AKT (C, by densitometry of western blots; n = 8; *p<0.05) and reduced p-eNOS/total eNOS (D, determined from western blots; n = 8; *p<0.05) ratios.

**Figure 3.** At baseline, there was no difference in expression of VEGF R 2 at both mRNA and protein levels (A and B). However, mice with diabetes showed a decreased expression of membrane bound VEGFR1 at both mRNA(C) and protein (D; integrated density of VEGFR1/actin from western blots; n = 8 *p<0.05) levels. Similarly, soluble VEGFR1 expression was downregulated in mice with DM at both mRNA (E) and protein (F, ratio of sVEGFR1/actin, determined from western blots; n = 8; *p<0.05) levels.

**Figure 4.** A. In response to acute ischemia, VEGF A level significantly increased (fold change from the non-ischemic contralateral limb muscles) in both control mice (NC, n =
6 per group; *p <0.01) and mice with diabetes (DM; n = 6 each group; *p <0.01), but there was no difference between the two groups. B. The ratio of expression of phospho-AKT (p-AKT) to total AKT was measured as an index of VEGF A mediated signaling. In control mice, p-AKT to total AKT ratio was significantly higher at day 3 after ischemia (as determined from western blots; n = 8; *p < 0.05). However, muscles from diabetic mice showed a reduced p-AKT/total AKT ratio at baseline and there was no significant change in the ratio in response to ischemia (determined from western blots; n = 8; p > 0.05). C. Similarly, the ratio of phospho-eNOS (p-eNOS) to total eNOS was measured as another index of VEGF mediated signaling. In control mice, both p-eNOS and total eNOS were upregulated following ischemia, and there was no net change in the ratio of p-eNOS/total eNOS (densitometry of western blots; n = 4; p > 0.05). However, in mice with diabetes, the ratio of p-eNOS to total eNOS was significantly lower in the ischemic limb (n = 4; p <0.01).

NI: non-ischemic muscle; I: ischemic muscle.

**Figure 5.** At mRNA level (A), no difference in VEGF R2 expression was detected between ischemic and baseline samples in both groups. However, at protein level (B), VEGF R2 expression was increased in both control and diabetic groups in response to ischemia, and there was no significant difference between the groups (NC vs. DM, fold increase from non-ischemic limb, as determined by densitometry from western blots; n = 9; p>0.05). In response to ischemia, the DM group had a greater increase in expression of VEGFR1 compared to NC at both mRNA (C) and protein (D; fold increase from non-ischemic limb, determined by densitometry from western blots; n = 9; *p < 0.001) levels. The expression of sVEGFR1 significantly increased in both groups. However, the
magnitude of sVEGFR1 increase in mice with DM was much greater than that for NC group at both mRNA (E) and protein (F, fold increase from non-ischemic, determined by densitometry from western blots; n = 9; *p < 0.05) levels. NI: non-ischemic muscle; I: ischemic muscle.
TABLE 1, Primers and probes for real-time PCR and RT-PCR

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Figure 1
Figure 4.

A.

B.

C.