Abstract—Deficient angiogenesis after ischemia may contribute to worse outcomes of peripheral arterial disease in patients with diabetes mellitus (DM). Vascular endothelial growth factor (VEGF) and its receptors promote angiogenesis. We hypothesized that in peripheral arterial disease, maladaptive changes in VEGF ligand/receptor expression could account for impaired angiogenesis in DM. Skeletal muscle from diet-induced, type 2 diabetic (DM) and age-matched normal chow (NC)-fed mice was collected at baseline and 3 and 10 days after hindlimb ischemia and analyzed for expression of VEGF, full-length VEGF receptor (VEGFR)-1, soluble VEGFR-1, and markers of downstream VEGF signaling using ELISA, reverse transcriptase-polymerase chain reaction, and Western blots. In the absence of ischemia, DM mice had increased VEGF (NC versus DM: 26.6 ± 2.6 versus 53.5 ± 8.8 pg/mg protein; P < 0.05), decreased soluble and membrane-bound VEGFR-1 (NC versus DM: 1.44 ± 0.30 versus 0.85 ± 0.08 and 1.03 ± 0.10 versus 0.72 ± 0.10, respectively; P < 0.05), decreased phospho-AKT/AKT and phospho–endothelial NO synthase/endothelial NO synthase (NC versus DM: 0.76 ± 0.2 versus 0.38 ± 0.1 and 0.36 ± 0.06 versus 0.25 ± 0.04, respectively; P < 0.05), and no change in VEGFR-2. After ischemia, both DM and NC had comparable increases in VEGF-A. VEGFR-1 and soluble VEGFR-1 expression increased in both groups, but the fold increase was significantly greater in DM. These data demonstrate that soluble VEGFR-1, an angiogenesis inhibitor, is regulated in skeletal muscle by type 2 DM and ischemia. In the absence of ischemia, despite reductions in both soluble VEGFR-1 and VEGFR-1, VEGF ligand signaling is lower in DM compared with controls. After ischemia, maladaptive upregulation of these receptors further reduces the capacity of VEGF to induce an angiogenic response, which may provide a novel target for therapy. (Circ Res. 2007;101:948-956.)

Key Words: vascular disease • growth factors • nitric oxide

Angiogenesis, the formation of new blood vessels from preexisting ones, plays a significant role in many physiological and pathological conditions. Vascular endothelial growth factor (VEGF) and the corresponding VEGFR tyrosine kinases play a key role in blood vessel formation during development and in the regulation of angiogenesis in the adult. The 2 main VEGFRs are fms-like tyrosine kinase 1 (Flt-1 or VEGFR-1) and fetal liver kinase 1 (Flk-1 or VEGFR-2). In the adult, VEGF-A mediates angiogenesis primarily through VEGFR-2, whereas a role for VEGFR-1 is less clear, and genetic and cellular studies suggest that VEGFR-1 can negatively modulate the angiogenic effects of VEGFR-2. In addition, VEGFR-1 could bind VEGF and reduce the availability of VEGF to VEGFR-2. Soluble (s)VEGFR-1, a naturally occurring splice variant of VEGFR-1, lacks the transmembrane domain but binds VEGF with very high affinity and acts as an angiogenesis inhibitor by sequestering VEGF. Increased expression of sVEGFR-1 from the placenta has been linked to the hypertension and proteinuria in preeclampsia. Ambati et al demonstrated that sVEGFR-1 has a physiological role in the cornea to maintain avascularity. Although studies have established a role for VEGF-A in skeletal muscle angiogenesis, the effect of changes in receptor expression on the angiogenic response to ischemia is not known.
Several of the long-term complications of diabetes mellitus (DM) involve abnormalities in angiogenesis. Increased VEGF-mediated angiogenesis has been implicated in retinopathy and nephropathy, whereas there is an attenuated angiogenic response in wound healing and ulcers. In patients with cardiovascular disease, a defective angiogenic response to ischemia could, in part, account for poor clinical outcomes. In preclinical models of peripheral arterial disease (PAD), diabetic animals have been shown to have attenuated perfusion recovery in response to ischemia. Studies that have investigated the causes of the reduced angiogenic response in diabetes have focused on an impaired release of endothelial progenitor cells from the bone marrow, defective function of progenitor cells, or formation of advanced glycosylation end products. However, there has been no systematic examination of the role of changes in the VEGF–ligand system in this process.

Studies have shown higher levels of VEGF-A in the plasma of subjects with diabetes compared with controls. Sasso et al showed that expression of VEGF-A is increased in the myocardium of patients with type 2 diabetes along with decreased expression of VEGFR-1 and VEGFR-2 and evidence of reduced downstream VEGF signaling. Simply providing exogenous VEGF-A has met with little success in clinical trials. Taken together, blunted angiogenesis in patients with PAD and diabetes might be attributable to a VEGF signaling defect in which there is reduced receptor signaling despite higher ligand expression, similar to insulin resistance. Alternatively, reduced receptor function despite greater ligand expression could result from ligand trapping, which reduces the availability of free, functional ligand. This could occur with or without changes in VEGFR expression. This study was designed to test the hypothesis that alterations in the VEGFR–ligand system play a role in impaired skeletal muscle angiogenesis in type 2 DM.

Materials and Methods

Mouse Model of Type 2 DM

Type 2 DM was induced in male C57BL/6 mice (n=70; The Jackson Laboratory, Bar Harbor, Me) by feeding a high-fat diet for 14 weeks and was confirmed as described previously. Age-matched C57BL/6 mice (n=70) fed a normal chow (NC) diet served as controls. Protocols were approved by the Duke University Institutional Animal Care and Use Committee.

Experimental Design and Groups

A separate group (n=10 per group) was used for measurement of perfusion recovery after hindlimb ischemia. For tissue analysis, both control (NC) (n=60) and diabetic (DM) (n=60) mice were divided into 3 groups: baseline and days 3 and 10 postischemia (n=20 per time point). The tibialis anterior (TA) muscle was harvested and used for protein (n=10) and mRNA (n=10) analysis. In animals with unilateral hindlimb ischemia, the nonischemic contralateral muscles did not show any differences between day 3 and 10 time points; therefore, these groups were combined into 1 nonischemic group.

Surgical Induction of Hindlimb Ischemia and Hemodynamic Assessment

Unilateral hindlimb ischemia was induced and limb perfusion was measured as previously described. At each time point, results were expressed as a ratio of perfusion in the ischemic limb to that in the contralateral limb. The immediate postligation ratio was set as the baseline (day 0), and changes in perfusion were calculated as the difference in perfusion between the final time and the baseline.

RNA Analysis

Total RNA was isolated for reverse transcriptase-polymerase chain reaction (RT-PCR) or real-time quantitative RT-PCR, as described. Primers, sequences and amplification conditions used are shown in Table.

Protein and Histological Analysis

Total muscle protein lysates were prepared and protein concentrations measured as previously described. VEGF-A and p-AKT/AKT levels were determined by ELISA, as described. To isolate membrane-enriched protein fractions for detection of VEGFRs, total protein extracts were ultracentrifuged at 75,000g for 30 minutes at 4°C, and the pellet was resuspended in 50 µL of Tris buffer. Total cellular (30 to 50 µg) or membrane (15 to 20 µg) proteins were separated on 8% SDS–polyacrylamide gels and transferred to poly(vinylidene-fluoride) membranes, which were incubated with the following antibodies: rabbit polyclonal anti–phospho-AKT (p-AKT), phospho–endothelial NO synthase (p-ENOS), and phospho–extracellular signal-regulated kinase (p-ERK) (1:500) and anti–total AKT, eNOS, and ERK (1:1000; Cell Signaling, Beverly, Mass); rat monoclonal anti–VEGFR-2 (1:250; Chemicon, Boronia-Victoria, Australia); mouse monoclonal anti–VEGFR-1 (1:500); and anti-actin (1:1000; Sigma-Aldrich, St Louis, Mo). After detection by chemiluminescence, the results were quantified by densitometry using ImageJ (version 1.36b; NIH). Levels of sVEGFR-1 were determined by ELISA (R&D Systems, Minneapolis, Minn), and the results were confirmed by Western blots, which differentiated VEGFR-1 from sVEGFR-1. To compare inflammatory cell infiltrates between DM and NC, the total nuclei per millimeter squared in the 3-day ischemic tissue was measured in 3 separate ×400 fields on hematoxylin/eosin-stained paraffin sections.

Exogenous VEGF-A Binding Assay

To determine whether lysates from DM versus NC muscle differed in their ability to bind VEGF-A, VEGF-A concentrations were determined in muscle homogenates by ELISA before and after the addition of increasing concentrations of exogenous VEGF-A using the Mouse VEGF-A Immunoassay (R&D Systems). Samples were mixed 1:1 with diluent containing exogenous VEGF-A at a concentration of 0, 20, 60, or 80 pg/mL and incubated in a 96-well plate overnight at 4°C. The remainder of the assay was performed according to the recommendations of the manufacturer. Samples from DM mice were diluted 2-fold to adjust for differences in the initial VEGF-A concentrations.

Statistics

Statistical analysis was done using SPSS software (version 13.0; SPSS, Chicago, Ill). Data are expressed as the means±SEM. Comparison among groups was done using ANOVA followed by Fisher’s post hoc test. Repeated-measures ANOVA was done to assess improvement in perfusion over time within groups. Statistical significance was set at a probability value of <0.05.

Results

Diabetic Mice Have Impaired Perfusion Recovery After Hindlimb Ischemia

After 14 weeks of high-fat diet, C57BL/6 mice had significantly greater body weight (43.52±1.8 versus 28.2±1.6 g; P<0.05), higher fasting glucose levels (187.88±8.45 versus 135.79±5.46 mg/dL; P<0.05), and impaired glucose tolerance (area under the glucose tolerance test curve: 29.658±1200 versus 14.811±541 mg/dL·min; P<0.05) compared with age-matched NC-fed mice (n=70 per group). Laser Doppler perfusion imaging was performed before,
immediately after, and on days 3, 10, 20, and 30 after hindlimb ischemia (n=10 per group). Perfusion recovery was significantly attenuated in DM mice compared with NC mice on postoperative days 10, 20, and 30 (Figure 1). The change in the perfusion from postligation (day 0) to day 3 was similar in DM and NC, but the recovery from days 3 to 10 differed between groups, and this difference was maintained for the duration of the study.

Table. Primers and Probes for RT-PCR

<table>
<thead>
<tr>
<th>Mouse Primers and Probes</th>
<th>Sequences</th>
<th>PCR Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF-A</td>
<td>Forward: TACTGCTGTACCTCACCTCCACCATG&lt;br&gt;Reverse: TCACCTGATGGACTCTGCT&lt;br&gt;Probe: AATGTGGTCCCCAGGTGGCCAC</td>
<td>Real-time PCR (95°C, 15 seconds; 60°C, 1 minute; 40 cycles)</td>
</tr>
<tr>
<td>18S RNA</td>
<td>Forward: CGGCTACCACATCAAGGAA&lt;br&gt;Reverse: AGCCGCGTAATTCCAGC&lt;br&gt;Probe: TCGTGGACACAGACTTGACCTC</td>
<td>Real-time PCR (95°C, 15 seconds; 60°C, 1 minute; 40 cycles)</td>
</tr>
<tr>
<td>VEGF-A</td>
<td>Forward: CTGTGCAAGGCTGCTAAGC&lt;br&gt;Reverse: GTTCCGAAACCTGAGGAG</td>
<td>PCR (94°C, 30 seconds; 56°C, 30 seconds; 72°C, 1 minute; 30 cycles)</td>
</tr>
<tr>
<td>VEGFR-1</td>
<td>Forward: GTCAACAGATGTGCGAATTG&lt;br&gt;Reverse: TGACCTGATCGTCAGCAG</td>
<td>PCR (94°C, 30 seconds; 58°C, 30 seconds; 72°C, 1 minute; 30 cycles)</td>
</tr>
<tr>
<td>sVEGFR-1</td>
<td>Forward: GTCAACAGATGTGCGAATTG&lt;br&gt;Reverse: TGACTTTGTTGTGACAAATC</td>
<td>PCR (94°C, 30 seconds; 58°C, 30 seconds; 72°C, 1 minute; 30 cycles)</td>
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<tr>
<td>VEGF-2</td>
<td>Forward: AGAACACCAAAAGAGAGGAG&lt;br&gt;Reverse: GCACACAGGCAAAAACATAC</td>
<td>PCR (94°C, 30 seconds; 58°C, 30 seconds; 72°C, 1 minute; 30 cycles)</td>
</tr>
<tr>
<td>18S RNA</td>
<td>Forward: TAAGAACCGAGACTCTGGCAT&lt;br&gt;Reverse: CCGCACTCTAAGGCGACATCAG</td>
<td>PCR (94°C, 30 seconds; 58°C, 30 seconds; 72°C, 1 minute; 30 cycles)</td>
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Diabetic Mice Have Higher Expression of VEGF-A but Reduced VEGF Signaling

In the TA muscle from DM mice not subjected to hindlimb ischemia, expression of VEGF mRNA and protein was approximately 2-fold higher (Figure 2A and 2B) in DM versus NC. VEGF exerts many of its effects via the AKT and ERK1/2 (p42/p44 mitogen-activated protein kinase) pathways. The AKT pathway leads to downstream activation of...
eNOS and release of nitric oxide, which results in cGMP production. The ratios of p-AKT/total AKT and p-eNOS/total eNOS (Figure 2C and 2D) and cGMP concentrations (Figure 2F) were significantly lower in DM versus NC, indicating that despite increased VEGF expression, there is reduced downstream VEGF signaling. Interestingly, we found no difference in the phosphorylation state of ERK1/2 between DM and NC (Figure 2E).

**Diabetic Mice Have Reduced Expression of VEGFR-1**

In TA muscle from mice not subjected to hindlimb ischemia, expression of VEGFR-2 mRNA and protein did not differ between DM and NC (Figure 3A and 3B). In contrast, both mRNA and protein expression of full-length VEGFR-1 (Figure 3C and 3D) and its soluble form, sVEGFR-1 (Figure 3E and 3F), were significantly decreased in DM versus NC.

**Studies of the VEGF Ligand-Receptor System Following the Induction of Hindlimb Ischemia**

As shown in Figure 1, when compared with NC mice, mice with DM had attenuated perfusion recovery by day 10 after hindlimb ischemia, but the extent of recovery was similar between the 2 groups at the day 3 time point. We therefore investigated whether differences in VEGF protein or signaling were responsible for the differences in perfusion observed at these 2 time points. Consistent with results shown in Figure 2, in the nonischemic limb, VEGF protein was higher, whereas p-AKT/total AKT and cGMP were lower in DM versus NC (Figure 4A through 4C, left bars). At day 3 after ischemia, VEGF expression was significantly greater in the ischemic versus nonischemic muscle in both the NC and the DM mice (Figure 4A, middle); however, the magnitude of the change was similar in both groups (NC versus DM, 5.1±1.8- versus 4.2±1.4-fold, respectively). By day 10, VEGF protein expression was not significantly different in the ischemic versus nonischemic limb in both NC and DM mice (Figure 4A, right). In NC mice (Figure 5B), there was no difference in the ratio of p-AKT/total AKT in the ischemic versus nonischemic limb on either day 3 or 10 after ischemia. However, in DM, the p-AKT/total AKT ratio was significantly reduced in the ischemic limb compared with that in the nonischemic limb on both days 3 (~33%) and 10 (~55%) after ischemia.

**Figure 2.** VEGF signaling is reduced in diabetic mice at baseline. Compared with muscle from NC-fed mice, nonischemic TA muscle from mice with diabetes (DM) displayed increased expression of VEGF-A mRNA (A) and protein (B). Nonischemic DM muscle also displayed evidence of reduced VEGF signaling, as indicated by decreased p-AKT/total AKT (C), reduced p-eNOS/total eNOS (D), and reduced cGMP concentration (E). However, there was no change in the expression or activation of ERK1/2 (F).

*P<0.05 (n=5 to 8/group).
ischemia. In NC mice, cGMP levels were significantly greater at the day 3 and 10 time points when compared with the nonischemic limb (Figure 4C). In DM, cGMP levels in the ischemic limb were not significantly different from those in the nonischemic limb on day 3, but they were significantly increased by day 10 (Figure 4C). At all time points measured, the cGMP concentrations were significantly greater in NC versus DM mice.

**Differential Changes in VEGFR-1 and sVEGFR-1 Expression After Ischemia**

VEGFR-2 expression increased in both DM and NC mice at days 3 and 10 after hindlimb ischemia, but the magnitude of change was similar between the 2 groups (≈13.4- and ≈13.6-fold). In contrast, in NC mice, VEGFR-1 expression was not significantly different on days 3 and 10 when compared with the nonischemic limb (Figure 5A). However, in DM mice there was a significant increase in expression of VEGFR-1 (Figure 5A) at both days 3 and 10 after ischemia. The pattern of response for sVEGFR-1 was similar to that of VEGFR-1, but it was of an even greater magnitude (Figure 5B). Expression of sVEGFR-1 increased in both groups at days 3 and 10 after ischemia; however, the magnitude of sVEGFR-1 increase in DM (≈5- and ≈19-fold at days 3 and 10, respectively) was significantly greater than that in the NC group (≈3- and 7-fold at days 3 and 10, respectively) (Figure 5B). VEGFR-1 is also expressed on monocytes.30 However, the large difference in VEGFR-1 and sVEGFR-1 in DM could not be explained by a difference in inflammatory cell infiltrate between the 2 groups, because the total nuclei count in the ischemic tissue was similar in NC and DM (102 ± 10.2 versus 87 ± 15.2 nuclei/mm²; P = NS).

**Diabetic and Control Muscle Extracts Differ in VEGF Binding**

Nonischemic skeletal muscle from DM mice has higher expression of VEGF ligand and lower expression of sVEGFR-1 and full-length VEGFR-1 compared with NC mice (Figure 3F). We hypothesized that increased expression of VEGFR-1 and sVEGFR-1 would result in reduced VEGF availability. To test this possibility, we relied on the fact that the VEGF ELISA used in these studies does not detect VEGF when it is bound to sVEGFR-1 (Figure I in the online data supplement at http://circres.ahajournals.org). Therefore, in tissues with higher expression of sVEGFR-1, addition of exog-
enous VEGF ligand would result in a lower than expected increase in detectable VEGF protein. Addition of increasing amounts of exogenous VEGF to muscle homogenates from both groups resulted in a significantly greater increase in detectable VEGF in the DM muscle than in NC muscle (measured as the slope of the curve; Figure 6). The results were the same when dilutions where made to adjust for differences in the baseline concentration of endogenous VEGF because DM muscle had higher concentrations of VEGF (Figure 3F). These findings suggest that increased skeletal muscle sVEGFR-1 corresponds with a greater capacity to bind VEGF.

A favorable adaptation to ischemia would result in greater VEGF availability when ligand concentrations are increased. When we tested the effects of adding exogenous VEGF to 3-day postischemic muscle homogenates, the slope of the response line to exogenous VEGF in NC muscle increased ∼3.6-fold when compared with nonischemic muscle. In contrast to this favorable change, in samples from DM mice, the slope of the curve remained virtually identical (∼1.3-fold change versus nonischemic). These findings indicate that in DM, ischemia-induced increases in VEGF-A would not result in more VEGF being available when compared with the nonischemic state.

**Discussion**

The relative inability to mount a robust angiogenic response to ischemia following arterial occlusion may well contribute to the poor clinical outcomes observed in diabetic patients with coronary or PAD. Although a number of factors are likely to contribute to impaired angiogenesis in diabetes, the results of our study are the first to describe alterations in the VEGFR–ligand system as a potential contributor to this process. We and others found that mice with DM have
impaired perfusion recovery following femoral artery ligation and excision.\textsuperscript{15,28} Although VEGF-A and VEGFR-2 levels increased in both NC and DM groups in response to ischemia, mice with diabetes showed a proportionally greater increase in the expression of both sVEGFR-1 and full-length VEGFR-1. These alterations in the VEGFR–ligand system posts ischemia in DM mice would be predicted to, and indeed did, result in maladaptive alterations in VEGFR signaling, which were likely

Figure 5. Expression of VEGFR-1 and sVEGFR-1 is increased after hindlimb ischemia in diabetic mice. A, In response to ischemia, expression of membrane-bound VEGFR-1 was significantly increased in diabetic (DM) mice at both 3 days (3dl) and 10 days (10dl) but not in NC-fed mice. \( ^* P < 0.01 \) (n=6 to 9/group). B, Expression of sVEGFR-1 significantly increased in both groups following ischemia, but the increase in sVEGFR-1 in DM was significantly greater than in NC. \( ^* P < 0.05 \) (n=4 to 6/group). NI indicates nonischemic.

Figure 6. Increased skeletal muscle expression of VEGFR-1 and sVEGFR-1 corresponds with increased VEGF binding. To determine whether VEGFR-1 and sVEGFR-1 present in muscle tissue could bind and sequester VEGF-A, the concentration of VEGF-A in muscle homogenates was determined by ELISA before and after the addition of the indicated concentrations of exogenous VEGF-A protein. Samples from mice with diabetes (DM) were diluted to correct for the differences in baseline VEGF-A concentration from control (NC) mice. In the absence of ischemia, the higher baseline concentration of VEGFR-1 and sVEGFR-1 in NC muscle resulted in significantly lower levels of detectable VEGF-A, likely resulting from increased binding by VEGFR-1 and sVEGFR-1 (n=8/group). \( ^* P < 0.05 \).
protein tyrosine phosphatases PTP-1B and LAR and the ischemia. The fact that sVEGFR-1 is expressed at relatively high levels in normal vascular function to limit inappropriate vascular growth. However, given the apparent “VEGF resistance” in diabetes, increased VEGF and decreased sVEGFR-1 in nonischemic diabetic skeletal muscle are likely compensatory responses to maintain vascular function. In contrast, the changes in expression of VEGFR-1 and sVEGFR-1 following ischemia were not adaptive and may well have compromised the angiogenic response to ischemia in DM. These findings support the idea of targeting sVEGFR-1 to modulate ischemic angiogenesis in diabetes.

We also found that DM and ischemia alter the ratio of VEGFR-2 to VEGFR-1. VEGF-mediated angiogenic responses have been linked primarily to VEGFR-2. Thus, whereas a role for VEGFR-2 in postnatal physiologic angiogenesis is clear, the role of VEGFR-1 is less certain. The vascular phenotype of VEGFR-1 knockout mice suggests that VEGFR-1 negatively regulates endothelial cell proliferation. In addition, mice expressing a kinase domain-deficient mutant of VEGFR-1 undergo normal vascular development, indicating that at least 1 role for VEGFR-1 is to trap excess VEGF-A ligand and regulate its availability for VEGFR-2. Consistent with this possibility, the affinity of VEG-F-A for VEGFR-1 is ~10-fold higher than its affinity for VEGFR-2. Furthermore, VEGFR-2-mediated endothelial cell proliferation is suppressed by VEGFR-1, and embryonic stem cells lacking VEGFR-1 show increased phosphorylation of VEGFR-2. Other reports indicate that VEGF-induced migration of mesenchymal stem cells in vitro is mediated by VEGFR-1, thereby implicating VEGFR-1 in the recruitment of hematopoietic stem cells in ischemic and tumor angiogenesis. In light of these observations, VEGFR-1 may exert a dual effect on angiogenesis, as a negative modulator through its effects on VEGF and VEGFR-2 and as a positive modulator by recruiting hematopoietic stem cells.

The fact that expression of membrane-bound VEGFR-1 paralleled that of sVEGFR-1 in both diabetes and ischemia is consistent with the fact that these proteins result from alternative splicing of the same mRNA and with the possibility that these 2 proteins perform similar functions. Importantly, however, it remains unclear whether specific transcriptional regulatory mechanisms differentially regulate sVEGFR-1 expression. Collectively, our data suggest that the downregulation of VEGFR-1 and sVEGFR-1 in baseline diabetic skeletal muscle may be beneficial to maintain vascular homeostasis in the setting of decreased VEGF sensitivity. Following ischemia, the greater increase in VEGFR-1 and sVEGFR-1 may limit the ability of VEGF to bind to VEGFR-2, resulting in decreased angiogenesis. Our data leave open the possibility that exogenous VEGF could act therapeutically if it were available in a quantity sufficient to overcome the endogenous blocking by receptors, a possibility supported by a recent study from our group. These findings also suggest that other targets within the system that lead to altered VEGF signaling may be equally, if not more, beneficial.

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Disclosures

None.

References


Impaired Angiogenesis After Hindlimb Ischemia in Type 2 Diabetes Mellitus: Differential Regulation of Vascular Endothelial Growth Factor Receptor 1 and Soluble Vascular Endothelial Growth Factor Receptor 1
Surovi Hazarika, Ayotunde O. Dokun, Yongjun Li, Aleksander S. Popel, Christopher D. Kontos and Brian H. Annex

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Supplementary Figure 1. To determine whether the VEGF-A ELISA (R&D systems, Minneapolis) used in this study can detect VEGF-A when bound to soluble VEGFR-1 (sFlt-1), we did standard curves of recombinant VEGF-A in absence of sFlt-1, and in presence of increasing concentrations of sFlt-1 (final sFlt-1 concentrations of 2, 4 and 8 ng/ml). The slope of the standard curve decreased with increasing concentrations of sFlt-1, indicating that the assay detects primarily free VEGF A.