Skeletal Muscle VEGF Gradients in Peripheral Arterial Disease: Simulations of Rest and Exercise

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ABSTRACT

Vascular endothelial growth factor (VEGF) is a key promoter of angiogenesis and a major target of pro-angiogenic therapy for peripheral arterial disease (PAD). Greater understanding of VEGF angiogenic signaling and guidance by gradients for new capillaries will aid in developing new pro-angiogenic therapies and improving existing treatments. However, in vivo measurements of VEGF concentration gradients at the cell scale are currently impossible. We have developed a computational model to quantify VEGF distribution in extensor digitorum longus skeletal muscle using measurements of VEGF, VEGF receptor (VEGFR), and neuropilin-1 (NRP1) expression in an experimental model of rat PAD. VEGF is secreted by myocytes, diffuses through and interacts with extracellular matrix and basement membranes, and binds VEGFRs and NRP1 on endothelial cell surfaces of blood vessels. We simulate the effects of increased NRP1 expression and of therapeutic exercise-training on VEGF gradients, receptor signaling and angiogenesis. Our study predicts that angiogenic therapy for PAD may be achieved not only through VEGF upregulation, but also through modulation of VEGFRs and NRP1. We predict that: expression of $10^4$ NRP1/cell can increase VEGF binding to receptors by 1.7-fold (versus no NRP1); in non-exercise-trained muscle with PAD, angiogenesis is hindered due to limited VEGF upregulation, signaling and gradients; in exercise-trained muscle, VEGF signaling is enhanced by upregulation of VEGFRs and NRP1, and VEGF signaling is strongest within the first week of exercise therapy; and that hypoxia-induced VEGF release is important to direct angiogenesis towards unperfused tissue.
INTRODUCTION

Peripheral Arterial Disease (PAD) is a disorder affecting 15% of adults over 55; it results in insufficient blood flow during exercise (intermittent claudication) or rest. Patients with PAD have a 25% higher mortality rate and risk amputation (50,000 cases/year in the United States).(6, 62) Currently, rehabilitative exercise training is regarded as the most effective treatment for intermittent claudication, but is only applied to select patients and its therapeutic mechanisms are poorly understood.(46)

New therapies for PAD and pre-clinical models of PAD are generating intense interest.(62) The most common animal model of PAD is surgical ligation of one or both of the femoral or iliac arteries (single or double ligation). Though neither method completely reproduces the symptoms of PAD (and they are a more acute pathology than the chronic course of human PAD), they are valuable tools for studying PAD in several animal species including rat, mouse and rabbit.(3, 28, 41, 43, 47, 53) Following ligation, the affected leg loses blood flow (becomes unperfused) due to functional loss of a major artery, but blood re-perfusion begins within the first two weeks due to collateralization (growth and dilation of smaller arteries and vessels).(4, 16) Blood flow and tissue oxygen content partially recover to acceptable levels at rest, but limb activity is hindered and blood perfusion during exercise cannot reach pre-ligation levels.(3, 14, 41) It is this animal model of PAD (femoral ligation) for which we create a computational model in this study.

Vascular endothelial growth factor (VEGF) is a key promoter of both physiological and pathological angiogenesis. It increases proliferation and migration of endothelial cells and is widely studied in vivo and in vitro.(8, 18, 54, 55, 60) VEGF acts as a chemoattractant and directs capillary growth, so angiogenic response to VEGF depends on both VEGF concentration and
extracellular gradients of VEGF.(12, 15) In PAD, delivery of VEGF via bolus injection, adeno-associated viral vector or transplantation of pro-angiogenic cells has shown great therapeutic potential by increasing blood perfusion and tissue oxygenation, but each mode of delivery requires further investigations to confirm its effectiveness and safety.(9, 19, 56) An alternative pro-angiogenic therapy is exercise training, which has promising results and a low potential for harmful side effects.(3, 24, 62) However, more investigation is needed to better understand VEGF-induced angiogenesis and its application to PAD.

A variety of cells in the body, including myocytes (skeletal muscle fibers) secrete VEGF at different rates.(65) Five main splice variant proteins are produced from the VEGF-A gene, and isoforms 120 and 164 (corresponding to the number of amino acids) are the most frequently expressed.(25, 40, 48) VEGF\textsubscript{120} and VEGF\textsubscript{164} are both homodimeric glycoproteins but only VEGF\textsubscript{164} contains a domain that allows it to bind to neuropilin-1 (NRP1) co-receptors expressed on endothelial cell surfaces and to heparan sulfate proteoglycans (HSPG) present in high concentrations in the extracellular matrix (ECM) and basement membranes (BM) of the interstitial space. VEGF signaling occurs by VEGF binding to receptor tyrosine kinases VEGFR\textsubscript{1} and VEGFR\textsubscript{2}, causing the two VEGF receptors (VEGFRs) to signal intracellularly;(55) the receptor-ligand complex may also be internalized, thus removing VEGF from the interstitial space. The co-receptor NRPI modulates VEGF binding to receptors due to its ability to form VEGF\textsubscript{164}-VEGFR\textsubscript{2}-NRP1 and VEGF\textsubscript{120}-VEGFR\textsubscript{1}-NRP1 complexes (Figure 1).(55)

Hindlimb arterial ligation causes large areas of tissue to become unperfused, leading to hypoxia.(47) In normal muscle, hypoxia is detected by the oxygen sensing transcription factor, Hypoxia Inducible Factor (HIF) 1\(\alpha\), which stimulates VEGF upregulation.(58) In ligated rats, the
response to hypoxia changes, and expression of VEGF, VEGFRs and VEGF co-receptor NRP1 is different for exercise-trained versus non-trained rats.\(^{(28)}\) To gain better understanding of angiogenesis in PAD and the therapeutic angiogenic effect of exercise training, we must study the interaction of VEGF with both blood perfused and unperfused muscle in both exercise-trained and non-exercise-trained animals during rest and exercise throughout the course of PAD.

The interactions of VEGF ligands with their receptors and co-receptors are very complex, especially when spatial gradients are present, and it is impossible to achieve a quantitative understanding of this system using experimental methods alone. Computational/systems biology is emerging as a powerful methodology for studying complex systems from the molecular to tissue and organ levels. We have previously constructed models studying VEGF interactions with receptors in normal rat extensor digitorum longus (EDL) and in human vastus lateralis muscle,\(^{(29-31, 33)}\) and interactions with NRP1 and placental growth factor (PIGF) \emph{in vitro}.\(^{(32, 34)}\) In this study, we constructed a computational model to investigate the biochemical interactions and transport of VEGF \emph{in vivo} using the well-characterized hindlimb muscle in rat as a sample environment. We use an empirical relationship between VEGF secretion and oxygen concentration consistent with experimental data on HIF1\(\alpha\) response to oxygen combined with data on VEGF secretion in response to changes in HIF1\(\alpha\).\(^{(22, 58)}\) This is the first computational model of VEGF transport \emph{in vivo} under conditions of PAD in exercise-trained and non-exercise-trained rat that can predict VEGF distribution at spatial resolutions currently impossible to achieve experimentally. Use of this model will aid in understanding of the effect of NRP1 on VEGF gradients and of the impact of exercise training therapy for PAD. The model allows the analysis of exercise-induced VEGF and VEGF receptor upregulation over a 14 day period leading up to vascular remodeling, including hypoxia-induced VEGF upregulation, formation of
VEGF gradients, and angiogenic activation of endothelial cells by VEGF signaling. Data from femoral ligation experiments will be used for the upregulation of VEGF and its receptors during simulated PAD and exercise. This model is also general enough to be applicable to other tissue types in the future.

METHODS

We have constructed a 400×208×800 µm³ three-dimensional model of rat EDL muscle (including muscle fibers, microvessels, extracellular matrix and basement membranes) based on experimental observations (Figure 1).(3, 5, 11, 18, 23, 36-38, 58, 59) This three-dimensional model is used in calculations of blood flow and oxygen distributions, from which VEGF secretion from the myocytes is estimated as described below. However, owing to the complexity of the system and the number of the parameters that are of physiological interest that need to be explored, we choose a two-dimensional tissue cross section (400×208 µm³) in which we will characterize the VEGF gradients (Figure 2). Using empirical relationships for viscosity and hematocrit distribution,(21, 42) and blood oxygenation and velocity measurements in ligated rat EDL as parameters,(13, 14, 41, 42) we simulated blood flow for both fully perfused tissue (FPT) and partially perfused tissue (PPT) where only half of the tissue arterioles have blood flow (Figure 2). In vivo, there are heterogenous blood flow distribution in tissues, and some parts of a tissue are reperfused before others. Thus, in both the FPT and PPT, perfusion returns over the course of 14 days. They are both models of femoral ligation and subsequent perfusion recovery, for different parts of the muscle experiencing different blood flows.

As in a previous study,(21) we used blood flow simulation results to perform oxygen transport simulations for resting and exercising conditions (Figure 2).(2, 18, 21) Using
experimental data on the dependence of VEGF release on HIF1α and O2,(22, 28, 45, 58) we formulated an empirical relationship between oxygen tension and VEGF release at the level of a single myocyte (Figure 3). Finally, using parameters for expression and transport of VEGF,(10, 27, 34, 35, 40, 51, 52, 58, 65) expression and binding kinetics of VEGFRs and NRP1,(10, 28, 32, 34, 52, 63) and the simulated oxygen distributions, we performed VEGF transport simulations in a two-dimensional slice of EDL tissue for rat with surgically ligated artery upstream (as a model of PAD).

The time-course changes in blood oxygen content in ligation/PAD are based on experimental measurements for blood oxygenation and tissue oxygenation. In non-diseased tissue, blood oxygen saturation of arterioles feeding into capillaries (SO₂A) is 0.6 to 0.8.(20, 61) Although SO₂A has never been directly measured in skeletal muscle with PAD, tissue PO₂ measurements and values of hemoglobin saturation estimated from the PO₂ data show an immediate decrease in overall hemoglobin saturation following arterial ligation with a steady recovery over 14 days.(13, 14, 41) Therefore, SO₂A values of 0.06, 0.3 and 0.6 are used for 2, 7 and 14 days following ligation, respectively. These lower upstream oxygen contents are used to model the slow return of oxygenating flow to the tissue.

While non-exercise-trained rats are assumed to have no VEGFRs or NRP1 upregulation based on experimental measurements, for exercise-trained rats we performed VEGF transport simulations using the derived O₂-VEGF relationship and experimentally measured VEGFR and NRP1 expression upregulation levels for 2, 7 and 14 days after ligation for resting and exercising muscle.

The time period in this study ends at 14 days. We assume that vascular remodeling is limited within this time; after this, varying amounts of vascular remodeling would take place in
exercise treated and untreated muscles. These VEGF-dependent changes to the vascular networks would necessitate a feedback loop in which the blood flow and tissue oxygen levels are recalculated, having a follow-on effect on VEGF release and VEGFR binding. Such an iterative process can be implemented, but comprehensive, consistent quantitative data are not presently available on how the endothelial cells respond to the VEGFR binding and VEGF gradients, and thus this is beyond the scope of this study.

An expanded Materials and Methods section containing the description of the model geometry, complete governing equations, model parameters, and details of the numerical solution is available in the online data supplement at http://ajpheart.physiology.org/. The development of the kinetic network of interactions between the VEGF ligands and the VEGF receptors on the cell surface (33), and the development of the extensor digitorum longus model (29-31) have taken place incrementally over several publications.

RESULTS

Three-Dimensional Simulations of Oxygen Distribution in Hindlimb Ischemia

In PAD, ischemic tissue maintains normal oxygen consumption, but blood flow in microvessels becomes more heterogeneous and only some arterioles remain perfused. During and after the collateralization process, the unperfused tissue becomes hypoxic and angiogenesis takes place, resulting in the increase of capillary-to-fiber ratios in the tissue.(13, 38, 41) We performed simulations to study the effect of blood flow loss in regions of unperfused tissue, and the effect of light exercise on oxygen distribution in FPT and PPT.

In FPT with normal blood oxygenation (i.e., inlet arteriole SO$_2$ (SO$_2$A) is 0.6) the entire tissue remains well oxygenated (PO$_2$ > 22 mmHg) (Figure 2A). During light exercise, tissue oxygen consumption is assumed to be 6-fold resting level and capillary blood flow velocity
increases 180%. (3) No regions of extreme hypoxia (PO$_2$ < 1 mmHg) exist but the minimum PO$_2$ level decreases to 3 mmHg (Figure 2C).

In PPT, at rest, oxygen diffuses a minimum of 80 µm from the perfused region into the unperfused region, so only 34% of the unperfused region is hypoxic (Figure 2B). During light exercise, the perfused region can no longer oxygenate the unperfused region, so the entire unperfused region becomes hypoxic (Figure 2D). These results and other oxygen simulations are used in the following sections to predict VEGF secretion rate from individual muscle fibers.

Two-Dimensional Simulations of VEGF Distribution for Different Levels of Neuropilin-1

Absolute concentration of NRP1 has not been measured in EDL or any other muscle, but in explanted human endometrium, NRP1 mRNA is 1 to 2-fold VEGFR2 levels. Extrapolating this ratio we would expect approximately $10^4$ NRP1/cell in accordance with our calculated concentrations of VEGFR2 in skeletal muscle. (29, 44) Hence, we simulate uniform secretion of VEGF from muscle fibers and express 0, $10^3$, $10^4$ or $10^5$ NRP1/cell. Altering NRP1 expression changes interstitial unbound VEGF concentration, so we alter VEGF secretion rates accordingly to obtain the experimentally observed unbound VEGF concentration of 1 pmol/L. (17) The results are presented in Table 1 and Figure S1 (see supplementary material, http://ajpheart.physiology.org/).

VEGF gradients are measured relative to the average unbound VEGF concentration in the interstitial space (relative gradients). They are calculated as the local change in VEGF concentration across 1 µm of interstitial space, and are reported as change in VEGF concentration across 10 µm, divided by mean VEGF tissue concentration. 10 µm is chosen as a reasonable scale for chemotactic sensing. During sprout formation, a tip cell with a length of 30-
50 µm is leading the sprout (12, 50) and thus the tip cell could experience gradients several times larger than the values reported per 10 µm.

Average VEGF gradients increase from $2.9 \pm 3.6$ to $10.2 \pm 11.4 \%\text{VEGF/10µm (± SD)}$ when $10^5$ NRP1/cell is expressed (versus no NRP1 expression). Gradients are mainly due to the heterogeneous placement of the capillaries. VEGF concentration is lowest in regions of high capillarity because receptors on capillary cell surfaces are internalized and VEGF is removed from the interstitial space (Figure S1). NRP1 thus modulates gradients by changing receptor binding activity. Expression of $10^5$ NRP1/cell increases VEGF$_{164}$ binding to VEGFR2 (78 to 839 molecules/cell) by the formation of VEGF$_{164}$-VEGFR2-NRP1 complexes and decreases VEGF$_{164}$ binding to VEGFR1 (229 to 2 molecules/cell) by the formation of VEGFR1-NRP1 complexes. Also, the relative amount of unbound VEGF$_{120}$ versus unbound VEGF$_{164}$ increases (8% to 21%) when $10^5$ NRP1/cell are expressed. For lower NRP1 expression ($10^4$ NRP1/cell), gradients are not greatly affected ($2.9 \pm 3.6$ versus $3.3 \pm 3.9 \%\text{VEGF/10µm}$) because increased VEGFR2 binding is balanced by decreased VEGFR1 binding.

Two-Dimensional Simulations of VEGF Distribution for Arterial Ligation: Non-Exercise-Trained Rat

For non-exercise-trained rats with ligated arteries, no consistent trend in receptor expression can be observed from measurements, and different observations are reported on whether VEGFR1 is up- or downregulated in ischemia.(28, 38) However, the evidence agrees that change in VEGFR1 and VEGFR2 expression in rat is less than 70%, so we perform simulations of VEGF distribution assuming no changes in VEGFR expression for fully and partially perfused muscle.
Experimental measurements suggest that unlike healthy tissue, where decreasing environmental oxygen alone is sufficient to double VEGF mRNA expression, muscle with PAD (or ligated artery) upregulates VEGF (mRNA and protein) less than 50% even in the hypoxic environment shortly after ligation. We assume that uniform secretion (with $10^4 \text{NRP1/Cell}$) is representative of EDL in ligated hindlimb (Figure S1C). VEGF gradients average 3.3 ± 3.9 %VEGF/10µm and maximum VEGFR2 binding is 236 molecules/cell.

Two-Dimensional Simulations of VEGF Distribution for Arterial Ligation: Exercise-Trained Rats during Exercise

In exercise-trained rats with PAD, mRNA expression of VEGF, VEGFRs, and NRP1 can be upregulated several fold (Figure 4). For these muscles, we simulate and compare VEGF transport in FPT and PPT using our oxygen-dependent model of VEGF secretion (Figure 3) and measurements for VEGFR and NRP1 expression for exercise-trained rats with femoral ligation. Control conditions assume normal blood oxygenation ($\text{SO}_2A=0.6$) and basal tissue receptor expression for both PPT (Figures 4,6A) and FPT (Figure S2, Figure 6A). VEGF gradients are similar (3.6 ± 4.1 versus 3.7 ± 4.1 %VEGF/10µm average, FPT versus PPT) but maximum VEGFR2 binding is higher in the unperfused region of PPT (1420 versus 961 molecules/cell). Two days following ligation, blood flow oxygenation is severely impaired ($\text{SO}_2A=0.06$), and our simulations predict that the tissue becomes completely hypoxic. Using Equation 1 (Online Supplement), we predict that fibers will uniformly secrete VEGF at 6-fold resting for both FPT and PPT, so no difference in VEGF transport is expected. VEGFRs are
upregulated (Figure 4), which allows VEGFR2 to bind more VEGF (1810 molecules/cell maximum) and increases VEGF gradients (11.3 ± 12.4 %VEGF/10µm).

Seven days after ligation, VEGFR upregulation and blood oxygenation increases (SO$_{2A}$=0.3). Average VEGF gradients increase (14 ± 15.8 and 14.2 ± 15.9 %VEGF/10µm in FPT and PPT, respectively) and maximum VEGFR2 binding remains elevated (1620 and 1820 molecules/cell in FPT and PPT, respectively). Two weeks after ligation, significant angiogenesis may have occurred, but we assume that the initial vessel network remains unchanged to compare VEGF gradients and VEGFR binding to the first week following ligation. Blood oxygenation is restored (SO$_{2A}$=0.6) so FPT is no longer hypoxic and VEGFR upregulation is reduced. In FPT and PPT, average VEGF gradients decrease to 5.2 ± 5.9 %VEGF/10µm and maximum VEGFR2 binding is reduced (but higher in PPT: 1260 and 1520 molecules/cell for FPT and PPT, respectively).

In the first 2 weeks after ligation, the balance between VEGF, VEGFR and NRP1 upregulation maintains average interstitial unbound VEGF concentrations between 0.9 and 1.7 pmol/L (compared to 1 pmol/L for non-diseased resting muscle).

Two-Dimensional Simulations of VEGF Distribution for Arterial Ligation: Exercise-Trained Rats during Rest

Exercise-trained rats spend 22 hours/day at rest.(28) We simulated VEGF distributions using oxygen distributions for resting EDL to determine VEGF secretion, and the same parameters for VEGFR and NRP1 expression as exercise-trained rat during exercise. Here, the differences between FPT and PPT are more pronounced (Figures 5,6B).
Two days after ligation, both FPT and PPT are completely hypoxic, therefore no difference is expected between simulations during exercise and rest (Figure 4B). Seven days after ligation, VEGF gradients in both FPT (Figure 5A) and PPT (Figure 5C) are high (12.6 ± 14.5 and 12.3 ± 13.6 %VEGF/10µm, respectively). However, maximum VEGFR2 binding is much higher in PPT (1810 versus 332 molecules/cell). Two weeks after ligation, assuming no angiogenesis occurred, blood oxygenation is restored and VEGF gradients decrease in both FPT and PPT (5.3 ± 6.4 and 5.0 ± 5.9 %VEGF/10µm, respectively). However, maximum VEGF-bound VEGFR2 remains higher in PPT (1670 versus 322 molecules/cell).

DISCUSSION

Recovery from PAD requires formation of new, functional blood vessels by collateralization and angiogenic sprouting. Initiation of angiogenic sprouting and guidance of the sprouts are dependent both on the overall concentration of VEGF and on the VEGF gradients. The ability to modulate VEGF gradients and understand angiogenesis mechanisms would greatly aid research into therapies for PAD, but these gradients cannot be measured experimentally with present technology. The goal of this study was to quantitatively predict VEGF concentrations, gradients and receptor signaling in vivo. We have constructed an anatomically-detailed and biophysically accurate computational model of VEGF transport and interaction with its receptors based on the fundamental equations of mass balance and biochemical kinetics, and performed computer simulations using parameters obtained from the experimental literature. Our model integrates the molecular biology of VEGF with in vivo tissue physiology (including blood flow and oxygen transport) to predict how PAD, or its animal model equivalent, femoral artery
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ligation, changes VEGF distributions, and the beneficial potential of exercise-training during PAD.

We have developed two types of models for studying VEGF receptors in skeletal muscle in vivo. The model of VEGF gradient formation in rat extensor digitorum longus was previously explored without the inclusion of Neuropilin (29, 31). VEGF interactions with VEGF receptors and with Neuropilin in human vastus lateralis muscle have been studied without the inclusion of VEGF gradients (33). Here we include the Neuropilin interactions in the rat EDL model and study the effects of exercise training on VEGF gradients and VEGF receptor binding.

Experiments have not shown whether endothelial tip cells respond to HSPG-bound VEGF. We present VEGF gradients as changes in unbound VEGF concentration relative to average interstitial VEGF concentration. The relative gradients (%VEGF/10µm) of unbound and total (unbound plus HSPG-bound) VEGF are the same because the low fractional occupancy of HSPG (<1%) results in a linear relationship between unbound and HSPG-bound VEGF concentration. Absolute gradients of HSPG-bound VEGF are larger, as the average HSPG-VEGF concentration is predicted to be 50-100 times the free VEGF concentration (33) (due to the unavailability of data for the number and affinity of binding sites for VEGF in ECM in vivo, these predictions are based on parameters extrapolated from other growth factors, e.g. fibroblast growth factor (10)). This assumes that HSPG concentration is uniform throughout the non-basement membrane interstitial space, which may not be true. Furthermore, during angiogenesis, proteases can focally cleave and release HSPG-bound VEGF.(26) However, in this study, we present steady state VEGF distributions for a sample homogeneous interstitial space.

NRPI can have a significant effect on both VEGF gradients and VEGFR binding kinetics, hence NRPI is an important molecule for VEGF signaling and guidance in both healthy
and diseased states. Our simulations show that expression of $10^4$ NRP1/cell increases VEGFR2 binding by 170% and decreases VEGFR1 binding by 75%; expression of $10^5$ NRP1/cell increases average VEGF gradients by over 200% (versus no NRP1 expression). Therefore, upregulation of NRP1 in addition to upregulation of VEGF may be more effective than upregulation of VEGF alone. Furthermore, relationship between VEGFR binding and NRP1 expression is complex and dependent on relative concentrations of NRP1, VEGFR1 and VEGFR2. Hence, the basal concentration of NRP1 and changes in expression of NRP1 in healthy and diseased muscle are critical for understanding angiogenesis and the potential for NRP1 to be a target for pro-angiogenic therapy.

We do not include soluble VEGFR1 (sFlt1) in this study. There is currently no data on the relative amounts of soluble vs. membrane bound VEGFR1, and whether this ratio changes under conditions of PAD, or during exercise. While the presence of soluble VEGFR1 acting as a sequestering agent for VEGF will affect the transient changes in the binding of VEGF to membrane-bound VEGF receptors, at steady state there would be no change due to sequestration, similar to the effects of changing the extracellular matrix VEGF binding site density (33). Significant heterodimerization of membrane and soluble VEGF receptors would result in the formation of dummy receptors for VEGF that would affect VEGF signaling, but the propensity of soluble VEGFR1 to autodimerize (1, 49, 57) would limit this surface interaction. For the presence of such heterodimers to have an impact on the comparison between exercise trained and untrained rats, training would have to change the soluble:membrane VEGFR1 ratio. An increase in this ratio would lead to a decrease in signaling of both VEGFR1 and VEGFR2 as the monomeric receptor tyrosine kinases are not activated by the soluble VEGFR1 upon dimerization.
Plasma levels of VEGF are typically found to be in the 1 pM range, similar to the interstitial free VEGF concentration. In addition, the permeability of the microvasculature of adult skeletal muscle to macromolecules of the size of VEGF is not high. Thus, convective transport of VEGF out of the tissue across the endothelium is not expected to be a significant factor and is not included in this model.

In a mouse model of PAD, NRP1 expression doubles with exercise training (not measured in rat). Our model predicts that the upregulation of NRP1 and VEGFRs increases average VEGF gradients over 6-fold. In our model, we assume that NRP1 and VEGFR upregulation is uniform for all endothelial cells, but it is likely that expression of these receptors responds to microenvironmental cues. Non-uniform distribution of VEGFRs and NRP1 can elevate VEGF gradients to higher levels and more effectively direct angiogenic sprouts, as well as increase VEGFR binding and VEGF signaling on specific endothelial cells. The spatial distribution of NRP1 upregulation cannot be quantified with present technology to a resolution at the cellular scale required for this study, but imaging of endothelial tip cells can provide qualitative observations of heterogeneities of receptor upregulation and its impact on angiogenesis and VEGF guidance.

VEGF upregulation normally results from hypoxia and shear stress, and angiogenic response to hypoxia and shear stress can be synergistic. However, the correlation between shear stress and VEGF release has not been quantified in vivo and its mechanism remains to be elucidated, so we use an oxygen dependent model of VEGF secretion. The form of the O2-HIF1α-VEGF secretion rate relationship has not been defined in vivo. We assume a nonlinear relationship, where the induction of VEGF upregulation is skewed towards conditions of severe
hypoxia. This agrees with current experimental observations of oxygen dependent VEGF release, but further study is needed to quantify the relationship between O$_2$ and VEGF release in vivo. 

For PAD without exercise therapy, up to 16% of capillaries become unperfused in perfused regions of tissue, and the spatial distribution of these capillaries has not been measured. (3) In our analysis, we do not include stop-flow capillaries and we expect these unperfused capillaries to induce areas of local hypoxia adding to the heterogeneity of VEGF distribution.

In normal rat muscle, hypoxia is sufficient to drive VEGF upregulation in muscle. (58) However, in rats with femoral ligation/PAD, hypoxia alone cannot induce significant VEGF and VEGFR upregulation, evidenced by the lack of VEGF upregulation in already severely hypoxic tissue and experiments with HIF1$\alpha$ in mouse. (39) The VEGF upregulation is limited to a 50% increase and arterioles lose their ability to dilate in response to muscle contractions, which limits increased blood flow to relieve hypoxia. (28, 38) Our oxygen transport simulations show that even with 2-fold blood velocity achieved by exercise training, hypoxia becomes prevalent in the muscle for light exercise and it is unknown whether exercise therapy can increase blood flow to healthy levels for more intense exercise (up to 25-fold in extreme cases). (2) We also shown that restoration of oxygen dependent VEGF release will greatly promote VEGF signaling and gradients, and both are crucial to proper angiogenic growth. Chronic exercise or electrical stimulation partially restores the ability of diseased muscle to increase blood flow in response to muscle contraction and upregulate expression of VEGF and its receptors. (3, 37) Understanding the limited VEGF response to hypoxia whether due to HIF1$\alpha$ or downstream signaling is important to understanding PAD and may reveal targets for gene or protein therapy or improved exercise therapy protocol that can more effectively restore normal muscle response.
Our simulations predict that the VEGF response to hypoxia can be critical for PAD therapy because it can favor angiogenic growth in unperfused tissue. In our simulations for exercise-trained rat during activity and rest, we assumed no angiogenesis has occurred by day 14 to change the capillary network in our simulations, but this may not be the case. Predicting such angiogenic growth requires a different model, beyond the scope of the present study of VEGF distribution and VEGFR signaling. However, even with no new vessels, VEGFR2 binding and VEGF gradients on days 2 and 7 are higher than the values on day 14 for FPT during activity (1810 versus 1260 molecules/cell, 14 versus 5 %VEGF/10µm). This difference is expected to be more dramatic if new capillaries grow by day 14 because the improved muscle oxygenation by the new capillaries will lower VEGF secretion. Therefore, the majority of angiogenic events would be predicted to take place in the first week post-ligation. For PPT during exercise, although gradients are lower for day 14 (5 %VEGF/10µm), VEGFR2 binding for vessels in the unperfused region can reach up to 1680 molecules/cell, which is higher than the perfused region. VEGF gradients can reach as high as 26% VEGF/10µm in between the perfused and unperfused regions. This difference is even more apparent for tissue during rest for days 7 and 14 post-ligation. Maximum VEGFR2 binding is 4.5-fold higher in PPT than in FPT (Figures 5,6B).

We have not included in this model the ability of the vasculature to respond to the VEGF receptor binding and VEGF gradients with angiogenesis and other vascular remodeling (the time period of study ends before remodeling). That these vascular changes are highly dependent on the VEGF family is clear, but the quantitative relationships, including for example, the dependence of vascular guidance on relative or absolute VEGF gradients, is not known. A major goal of our model is to predict the size of VEGF gradients (relative and absolute) and the level of VEGFR signaling that can be achieved in rat skeletal muscle in normal, diseased and treated
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states. These predictions can be taken in parallel with experimental data of vascular changes to give a fuller picture of the outcome.

The results of our simulations also demonstrate that the scale over which the gradient holds may be important. For example, compare Figure 5B and 5D. In each case, the relative gradients are similar, however it is clear that in one case there is a consistent directional gradient across the tissue section. In the other (Figure 5B), the absolute gradients are lower due to lower average VEGF concentration, but also the gradients are more localized, and do not persist over long distances. This may be an important distinction for the sensing of gradients, for example by filopodia that can be up to 50-100 µm in length.

In summary, we have created a biophysically detailed model of VEGF transport in skeletal muscle of the PAD-model rat. The study shows that NRP1 is an extremely important co-receptor with a complex ability to significantly modulate VEGFR binding. We predict that in non-exercise-trained muscle with PAD, angiogenesis is hindered due to limited VEGF upregulation, signaling and gradients. When this muscle undergoes exercise training, VEGF signaling is enhanced by upregulation of VEGFRs and NRP1, and signaling is strongest within the first week of exercise therapy. If upregulation of VEGFRs and NRP1 are not uniform on endothelial cells (i.e. expression is higher on perfused capillaries), VEGF gradients and signaling would be further enhanced. We also predict that VEGF release in response to hypoxia is important for angiogenesis to occur following the first week of exercise therapy in order to direct angiogenesis towards unperfused tissue. The ultimate goal of VEGF based therapies for PAD is to initiate angiogenesis through VEGF signaling. Our study predicts that this may be achieved not only through VEGF upregulation, but also upregulation of VEGFRs and NRP1.
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DISCLOSURES

None.
### Table 1

Sensitivity Analysis of NRPI Expression on VEGF gradients and Signaling

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FIGURE LEGENDS

Figure 1. Schematic of skeletal muscle geometry and VEGF transport. A, Rat EDL muscle cross section. Muscle fibers (red) and capillaries (blue) are separated by interstitial space (white). Skeletal Muscle (SM) fibers are regularly spaced and hexagonally packed. B, Interstitial space near a capillary. Muscle fibers are surrounded by a muscle basement membrane (MBM); capillaries formed by endothelial cells are surrounded by an endothelial basement membrane (EBM). The extracellular matrix (ECM) lies between the EBM and MBM. VEGF diffuses throughout the interstitial space (ECM, EBM and MBM). C-D, Diffusion and binding: VEGF$_{120}$ and VEGF$_{164}$ are secreted from SM into MBM and diffuse through the interstitial space but only VEGF$_{164}$ binds to HSPG (jagged line) in each layer. Near the endothelial cell surface (in the EBM), VEGF can interact with VEGFR1, VEGFR2 and NRP1 (denoted 1, 2 and N respectively). VEGF$_{164}$-VEGFR2-NRP1 complexes can be formed when VEGF$_{164}$-bound NRP1 couples with unbound VEGFR2 or when VEGF$_{164}$-bound VEGFR2 couples with unbound NRP1. VEGF$_{120}$-VEGFR1-NRP1 and VEGFR1-NRP1 complexes can be formed when NRP1 couples with VEGF$_{120}$-bound or unbound VEGFR1. All VEGF-bound and unbound receptors can be internalized.

Figure 2. Oxygen tension in the muscle fibers during ischemia. Distribution of oxygen in the EDL at rest (A, B) or during light exercise (C, D) (6-fold basal level oxygen consumption by tissue), for fully perfused tissue (A, C), i.e. all vessels have normal blood flow, or for partially perfused tissue (B, D), i.e. half the blood vessels have no flow. The cross-sectional slice used for the two-dimensional analysis of VEGF transport is marked.
Figure 3. Myocyte hypoxia increases VEGF secretion. VEGF secretion rate depends on the average oxygen concentration in the fiber cross section. Secretion in hypoxic muscle fibers (<1 mmHg) is 6-fold that in well oxygenated muscle fibers (>20 mmHg). The curve graphically represents Equation 1 (Online Supplement).

Figure 4. Receptor upregulation increases VEGF gradients during recovery from ligation in exercise-trained rats: exercise conditions in partially perfused tissue. Oxygen (upper small panel), VEGF secretion (lower small panel) and VEGF distribution (main panel) simulations for partially perfused tissue (PPT) (i.e., half the blood vessels have no blood flow) during light exercise after arterial ligation in exercise-trained rats. A, Control simulation: no VEGFR or NRP1 upregulation, normal blood oxygenation, i.e. arteriole inlet SO₂ (SO₂₄) is 0.6. B, Two days after ligation: blood is poorly oxygenated (SO₂₄=0.06) and NRP1 and VEGFR are upregulated. C, One week after arterial ligation: blood oxygenation is partially restored (SO₂₄=0.3). D, Two weeks after arterial ligation: blood oxygenation is restored (SO₂₄=0.6) and VEGF gradients decrease to near control levels. No angiogenic growth is included in the model.

Figure 5. Perfused and unperfused tissue during recovery from ligation in exercise-trained rats: resting conditions. Panels as in Figure 4. Fully perfused tissue (FPT) (i.e. all blood vessels have normal blood flow, A-B) and partially perfused tissue (PPT) (i.e. half the blood vessels have no blood flow, C-D) during rest, one and two weeks after arterial ligation in exercise-trained rats. In FPT, tissue is well oxygenated (>18 mmHg), which minimizes VEGF secretion.
In PPT, VEGF binding to VEGFR2 is up to 5-fold higher after one week and 4.5-fold higher after two weeks compared to FPT. No angiogenic growth is included in the model.

**Figure 6. Distribution of VEGFR2 binding in perfused and partially perfused tissue.**

VEGFR2 binding is compared for fully perfused tissue (i.e. all blood vessels have normal blood flow) and partially perfused tissue (i.e. half the blood vessels have no blood flow) during light exercise (A) and rest (B) after arterial ligation in exercise-trained rat.
REFERENCES


Skeletal Muscle VEGF Gradients in PAD


49. **Roeckl W, Hecht D, Sztajer H, Waltenberger J, Yayon A, and Weich HA.**


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