ABSTRACT

Title: DNA SEQUENCE VARIATION IN THE PROMOTER REGION OF THE *VEGF* GENE: IMPACTS ON *VEGF* GENE EXPRESSION AND MAXIMAL OXYGEN CONSUMPTION

Steven John Prior, Ph.D., 2005

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Maximal oxygen consumption (Vo_{2max}) is inversely associated with cardiovascular and all-cause mortality and is responsive to aerobic exercise training. A portion of the increase in Vo_{2max} with aerobic exercise training can be attributed to an increase in skeletal muscle capillarity (i.e., angiogenesis), which contributes to increased blood flow and oxygen extraction in working skeletal muscle. One contributing factor to exercise-induced angiogenesis is vascular endothelial growth factor (VEGF), as it is an endothelial cell proliferation and migration factor that is upregulated by acute aerobic exercise. Significant variability has been observed in VEGF protein levels, *VEGF* gene expression, skeletal muscle capillarity, and Vo_{2max} before and after aerobic exercise training. Additionally, variability is found in the DNA sequence of the gene encoding VEGF. Variation in the *VEGF* gene has the ability to impact *VEGF* gene expression and VEGF protein level and because of the relationship between VEGF, angiogenesis, and Vo_{2max}, we hypothesized that variation

in the *VEGF* gene is related to *VEGF* gene expression in human myoblasts, plasma VEGF level, and Vo_{2max} before and after aerobic exercise training.

The present report shows that *VEGF* promoter region haplotype impacts *VEGF* gene expression in human myoblasts *in vitro*. It was also found that *VEGF* promoter region haplotype was associated with Vo_{2max} in older men and women before and after exercise training in a manner that is consistent with the results of the *VEGF* gene expression experiments. Additionally, we found that plasma VEGF level was not associated with *VEGF* promoter region haplotype, nor did plasma VEGF level correlate with baseline Vo_{2max} or ΔVo_{2max} with aerobic exercise training. To date, we are the first to report that *VEGF* promoter region haplotype impacts *VEGF* gene expression in human myoblasts and is associated with Vo_{2max}. These results have potential implications for aerobic exercise training and may also contribute to the understanding of the function of the *VEGF* promoter region in different cell types. Furthermore, these results may prove relevant in the study of pathological conditions which can be affected by angiogenesis, namely obesity, cancer, coronary artery disease, and peripheral artery disease.

DNA SEQUENCE VARIATION IN THE PROMOTER REGION OF THE *VEGF* GENE: IMPACTS ON *VEGF* GENE EXPRESSION AND MAXIMAL OXYGEN CONSUMPTION

By

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3'UTR: 3' untranslated region

5'UTR: 5' untranslated region

ALS: amyotrophic lateral sclerosis

AP-1: activator protein-1

AP-2: activator protein-2α

bp: base pairs

CMV: cytomegalovirus

DNA: deoxyribonucleic acid

ECM: extracellular matrix

ELISA: enzyme-linked immunosorbent assay

HIF-1: hypoxia-inducible factor-1

HIF-1α: hypoxia-inducible factor-1α

$HR_{reserve}$: heart rate reserve

HRE: hypoxia response element

HRT: hormone replacement therapy

HUVEC: human umbilical vein endothelial cell

HWe: Hardy-Weinberg equilibrium

kb: kilobase pairs

L-VEGF: long VEGF

LD: linkage disequilibrium

MMP: matrix metalloproteinase

mRNA: messenger ribonucleic acid

NRP1: neuropilin receptor

OGTT: oral glucose tolerance test

PBMC: peripheral blood mononuclear cell

PCR: polymerase chain reaction

Po_2: partial pressure of oxygen

RFLP: restriction fragment length polymorphism

SNP: single nucleotide polymorphism

SV40: simian virus

sVEGFR-1: soluble vascular endothelial growth factor receptor 1 (also known as sFlt-1: soluble fms-related tyrosine kinase 1)

tPA: tissue plasminogen activator

uPA: urokinase plasminogen activator

VEGF: vascular endothelial growth factor protein

***VEGF*:** the vascular endothelial growth factor gene

VEGFR1: VEGF receptor 1 (also known as flt-1: fms-related tyrosine kinase 1)

VEGFR2: VEGF receptor 2 (also known as flk-1: fetal liver kinase-1, or KDR: kinase insert domain containing receptor)

Vo_{2max}: maximal oxygen consumption

ΔVo_{2max}: change in maximal oxygen consumption

INTRODUCTION

Maximal oxygen consumption (Vo_{2max}) is inversely associated with cardiovascular and all-cause mortality[28,42,75,84,98,151], a relationship that has been well documented in a variety of populations. It is also well established that Vo_{2max} is responsive to aerobic exercise training, such that Vo_{2max} typically increases 15-30% after 3-9 months of training[55,79,122,138,139]. Concordantly, improvement in cardiorespiratory fitness has been shown to result in decreased risk of cardiovascular disease mortality and all-cause mortality[11,38].

The vasculature of human tissues plays an integral role in survival and function. This role becomes even more prominent in certain pathological conditions (e.g., coronary artery disease[112], peripheral artery disease[6,39,47], cancer[70,87,143], and obesity[30,137]) and physiological conditions (e.g., aerobic exercise[21,50]) as blood flow to tissues is often limited. Vo_{2max} can be affected by the vasculature as limitations in blood flow can limit oxygen transport (particularly under conditions of physiological stress), negatively affecting metabolic processes in these tissues[109]. This limitation is especially relevant in human skeletal muscle where blood flow, and thus oxygen supply, have been determined to be limiting factors in exercise capacity[116,146]. As the genesis of new vasculature has the ability to mediate physiological responses by increasing local circulation and oxygen supply[89,109], investigation of the mechanisms underlying this process is of significant clinical interest.

Angiogenesis

Angiogenesis is the formation of blood vessels from pre-existing vessels and vascular endothelial cells[119,123]. The formation of new vasculature is a critical phenomenon in the adaptation to aerobic exercise training because a contributing mechanism to the increase in Vo_{2max} with aerobic exercise training is an increase in skeletal muscle capillarity achieved by angiogenesis[21]. This is especially relevant since a substantial proportion of the increase in Vo_{2max} with training is attributed to increased oxygen extraction by the working muscle[122,125]. Increased oxygen extraction by trained skeletal muscle has been demonstrated in humans[115] and in other animals[58,153]. Angiogenesis can contribute to this increase in oxygen extraction by increasing the capillary surface area for diffusion, decreasing the average O_2 diffusion path length in skeletal muscle, and increasing red blood cell transit time through skeletal muscle[109].

Aerobic exercise training has been identified as a powerful angiogenic stimulus as several studies over the last 3 decades have shown increases in skeletal muscle capillarity after aerobic exercise training, with increases of up to ~30% in as little as 1-3 months of training[1,21,71,101,134]. Aerobic exercise training has been shown to stimulate angiogenesis through a few key mechanisms: hemodynamic stimuli[36,49,61,72,94], muscle contraction[65,119,123,154], and metabolic stimuli[23,52,67,95,135]. Metabolic stimuli such as low oxygen tension have significant effects on angiogenesis through the regulation of growth factor expression, as well as the expression of other factors and receptors[23,52,67,95,108,135].

Vascular endothelial growth factor

Angiogenesis is a complex process involving a number of molecules acting to stimulate growth, direct migration, and stabilize new vessels; the coordination of these molecules is critical to achieve functional increases in vascular supply[108]. Vascular endothelial growth factor (VEGF) has been identified as one of the key regulators of angiogenesis[44,86,140] because it plays a role in endothelial cell proliferation[85,119,123,128], mobilization[5,73,97], and migration [65,119,123].

VEGF protein is encoded by the *VEGF* gene (chromosome 6p12) which contains 8 exons separated by 7 introns. VEGF is expressed in numerous human tissues including skeletal muscle, and recent research indicates that VEGF is involved in the angiogenic response to aerobic exercise[20,53,89,101,118]. Several studies in humans[48,53,117,118] and in other animals[9,20,89,101] have confirmed that aerobic exercise induces a 2-fold to 6-fold increase in VEGF mRNA. To date, hypoxia has been the best studied regulator of *VEGF* gene expression in relation to aerobic exercise. Aerobic exercise has been shown to result in an oxygen tension low enough (~2-4 Torr[116,147]) to cause hypoxic induction of *VEGF* gene expression and mRNA stabilization[45,88,127,145]. Several studies have provided evidence that hypoxic induction of *VEGF* gene and protein expression is mediated through hypoxia inducible factor-1 (HIF-1) and its oxygen-sensing subunit HIF-1α[23,52,67,95,135] by upregulating *VEGF* gene transcription[67,140] and increasing the half-life of VEGF mRNA[67,86,88,140]. Considering these studies and the role of VEGF, there is a considerable body of evidence suggesting that VEGF plays an integral role in the angiogenic response to aerobic exercise training.

Variability and heritability of Vo_{2max}, skeletal muscle capillarity and VEGF expression

Significant variability is observed in Vo_{2max}[19] and the response of Vo_{2max} to aerobic exercise training[130], even among humans of the same age, sex, and race. Variability is also observed in overall skeletal muscle capillarity, where capillary to fiber ratio has been shown to range from 0.81-1.97 cap/fiber among similar groups of individuals[21,105,107,134]. Furthermore, variability among individuals in *VEGF* gene expression exists as Schultz et al.[126] have demonstrated a range of ~1-fold to 7-fold induction of VEGF mRNA expression in monocytes derived from 51 individuals with coronary artery disease. Interestingly, this group found that individuals exhibiting the greatest hypoxic induction of VEGF mRNA expression had greater myocardial collateral circulation development than those with lower hypoxic induction, indicating functional implications of variable *VEGF* gene expression[126].

While some proportion of the variability in the aforementioned traits can doubtlessly be attributed to non-genetic factors, there appears to be a significant contribution of genetic factors. Twin studies have revealed significant correlations of Vo_{2max} between sibling pairs[18,77,78] and additional research has provided heritability estimates for Vo_{2max} and the response of Vo_{2max} to aerobic exercise training as high as 59%[17,41] and 47%[17], respectively; though it is recognized that non-genetic familial influences also contribute to these heritability estimates[17]. While the genetic contribution to skeletal muscle capillarity has yet to be defined, investigators have argued that differences in capillarity among individuals can be attributed to both environmental factors (e.g. aerobic exercise training), and genetic factors[21,114]. The

heritability of *VEGF* gene expression has not been well studied, but at least two recent reports have demonstrated that polymorphisms within the *VEGF* gene affect *VEGF* gene expression in specific cell types *in vitro*[82,141], indicating a genetic contribution to *VEGF* gene expression.

DNA sequence variation in the *VEGF* gene

The investigation of genetic factors underlying physical traits has progressed through the study of so-called 'candidate genes' that are likely to play a role in a given physiological process based on their known function. *VEGF* is an important candidate gene for Vo_{2max} because of its role in aerobic exercise-induced angiogenesis and because the DNA sequence of the *VEGF* gene is polymorphic. The DNA sequence in the promoter region of human genes is known to bind enhancers and other regulators of DNA transcription and the 5' untranslated region (5'UTR) is known to regulate VEGF expression at the posttranscriptional level[63]. Likewise, the 3' untranslated region (3'UTR) of the *VEGF* gene has the ability to regulate translation as factors bind the 3'UTR to stabilize VEGF mRNA[88,102]. Therefore, variation in these regions of the *VEGF* gene may regulate *VEGF* gene transcription and VEGF mRNA translation, with potential effects on VEGF protein expression and Vo_{2max}.

Several polymorphisms have been identified in the *VEGF* gene and in the 3kb of its upstream (5') promoter region[22,114,141,149]. Six of these polymorphisms have been investigated and four polymorphisms have been associated with measures of *VEGF* gene or protein expression in select human tissues. The C936T single nucleotide polymorphism (SNP) in the 3'untranslated region (UTR) has been

associated with plasma VEGF levels, and the G-634C, G-1154A, and C-2578A (which is linked to an 18bp insertion/deletion polymorphism at position -2549[22]) polymorphisms in the promoter region have been associated with *VEGF* gene or protein expression in C6 glioma cells[82], GI-1 glioma cells[82], and peripheral blood mononuclear cells (PBMCs)[129,149].

Furthermore, the AGG, AAG, and CGC *VEGF* promoter region haplotypes (combinations of alleles at the -2578/-1154/-634 SNPs, respectively) have been associated with *in vitro VEGF* gene expression in MCF7 breast cancer cells[141] and GI-1 glioma cells[82] using reporter gene constructs. Lambrechts et al.[82] also found that the same *VEGF* promoter region haplotypes were associated with plasma VEGF levels in a sample of European patients with amyotrophic lateral sclerosis (ALS; also known as Lou Gehrig's disease).

While the effects of these *VEGF* genotypes and haplotypes have been demonstrated in specific cell types, they have not been investigated in skeletal muscle. It is currently unclear whether the findings in glioma cells, PBMCs, and breast cancer cells will translate to tissue with greater relevance for Vo_{2max} such as skeletal muscle, where different factors may regulate *VEGF* gene expression. Furthermore, the effects of the *VEGF* promoter region haplotypes have not been investigated in the context of the continuous promoter region sequence (i.e., the entire region encompassing the -2578, -1154, and -634 SNPs) without the presence of additional genetic variation. In one experiment, the region from 5' position -2468 to -1177 (containing the HRE: hypoxia response element) was absent in the experimental construct[82]; in another experiment, additional genetic variation was

present in the experimental construct[141]. It is not known whether the same results would have been observed if these experiments were performed with the continuous promoter region sequence and without additional genetic variation.

Purpose

The current knowledge of the aforementioned *VEGF* polymorphisms is insufficient to draw conclusions about the effects of *VEGF* genotypes and haplotypes on *VEGF* gene expression in skeletal muscle, plasma VEGF levels, and Vo_{2max} before and after aerobic exercise training. The purpose of this project was to investigate individual polymorphisms in the *VEGF* gene for association with Vo_{2max} before and after aerobic exercise training, as well as to investigate the -2578/-1154/-634 *VEGF* promoter region haplotype (chosen based on previous reports of effects on *VEGF* gene expression in different cell types) for effects on *VEGF* gene expression in cultured human myoblasts, and for association with Vo_{2max} before and after aerobic exercise training.

In addition, the utility of measuring circulating VEGF protein levels is currently unknown. Plasma VEGF level appears to correlate well with the efficacy of treatments directed at angiogenic targets in clinical trials[136] and plasma VEGF levels are viewed by some to be markers of angiogenic activity[76]. Two reports have indicated that plasma VEGF level is increased after acute exercise[59,81], indicating that skeletal muscle does secrete VEGF protein into the circulation, but the relevance, abundance, and localization of that protein is still unresolved. As little is known about how indicative plasma VEGF levels are of skeletal muscle *VEGF* gene or protein expression, or about the usefulness of plasma VEGF level as a predictor of

Vo_{2max} and ΔVo_{2max} with aerobic exercise training, we investigated whether a correlation exists between plasma VEGF level and Vo_{2max} as well as whether an association exists between plasma VEGF level and *VEGF* promoter region haplotype.

HYPOTHESES

Hypothesis 1: Vo_{2max} before and after exercise training differs among subjects with different genotypes at the -2578, -1154, -634, -7 and 936 *VEGF* gene polymorphisms.

Hypothesis 2a: *VEGF* gene expression (as quantified by luciferase activity) under normoxic conditions will differ among the 4 common *VEGF* promoter region haplotypes: AGG, AAG, CGG, and CGC (-2578, -1154, and -634 polymorphisms, respectively).

Hypothesis 2b: *VEGF* gene expression (as quantified by luciferase activity) under hypoxic conditions will differ among the 4 common *VEGF* promoter region haplotypes: AGG, AAG, CGG, and CGC (-2578, -1154, and -634 polymorphisms, respectively).

Hypothesis 3: Based on the *VEGF* gene expression results under <u>hypoxic</u> conditions, Vo_{2max} before and after aerobic exercise training and ΔVo_{2max} with training will be lower in subjects with the AGG and/or CGG *VEGF* promoter region haplotypes than in subjects with the AAG and/or CGC haplotypes.

Hypothesis 4: Based on the *VEGF* gene expression results under <u>normoxic</u> conditions, plasma VEGF level before aerobic exercise training will be higher in subjects with only the CGG *VEGF* promoter region haplotype than in subjects with the AAG, AGG and/or CGC haplotypes.

METHODS

Subjects & Screening

Subjects for the study of Vo_{2max} and plasma VEGF protein levels were recruited to participate in a study investigating the effects of aerobic exercise training on lipoprotein-lipid levels and blood pressure. Direct-mail recruiting and media advertisements were used to recruit subjects. Respondents were then contacted by telephone to determine their initial eligibility for participation in the study. Written informed consent was obtained from all subjects at their first laboratory visit, after the entire study and its risks had been discussed and all of the subjects' questions were answered. Consent was obtained by the Principal Investigator, the Study Coordinator, or a qualified Research Assistant. The Institutional Review Board at the University of Maryland, College Park has approved the study protocol and consent form (IRB #00494 and #00736, P.I.: James M. Hagberg, Ph.D.).

To be admitted into the study, subjects were required to: 1) be sedentary (regular aerobic exercise less than 2 times per week and less than 20 minutes per session), 2) be 50-75 years of age, 3) not be taking lipid- or glucose-lowering medication, 4) be normotensive (systolic blood pressure less than 120mmHg and diastolic blood pressure less than 80mmHg) or hypertensive (systolic blood pressure between 121-160mmHg and/or diastolic blood pressure 81-99mmHg) controlled by medications, 5) have no recent history of smoking tobacco, 6) not have diabetes mellitus, 7) have no history of cardiovascular disease, 8) have a body mass index less than $37kg/m^2$, and 8) not have any other medical condition that would preclude vigorous aerobic exercise. Additionally, all female participants were at least 2 years

past menopause and agreed to maintain their hormone replacement therapy (HRT) status (currently taking or not taking HRT) for the duration of the study.

The subjects underwent two screening visits to confirm study eligibility. At the first screening visit, subjects had fasting blood samples drawn and underwent an oral glucose tolerance test (OGTT). Study eligibility requirements dictated that fasting plasma glucose levels were less than 126mg/dL and that 2-hour plasma glucose levels were less than 200mg/dL. Additionally, subjects had 20mL of blood drawn at this visit for DNA isolation and subsequent genotyping. At the second screening visit, subjects underwent a physical examination by a physician to detect conditions that would preclude aerobic exercise training[3] and a Bruce maximal treadmill exercise test to ensure that they had no evidence of cardiovascular disease[25]. Study eligibility requirements dictated that subjects exhibited less than 2mm of ST-segment depression and no signs or symptoms of cardiovascular disease during the treadmill test.

Baseline Vo_{2max} data were available for 196 subjects: 46 black men and women (n=13 and n=33, respectively) and 150 white men and women (n=66 and n=84, respectively). After 24 weeks of aerobic exercise training, Vo_{2max} data were available for 150 subjects: 30 black men and women (n=10 and n=20, respectively) and 120 white men and women (n=53 and n=67, respectively). Baseline plasma VEGF level was measured in 92 subjects: 35 black men and women (n=10 and n=25, respectively) and 57 white men and women (n=30 and n=27, respectively).

Exercise training intervention

Subjects underwent 24 weeks of standardized aerobic exercise training. The initial training volume was set to 3 sessions of 20 minutes at 50% of heart rate reserve ($HR_{reserve}$) per week. $HR_{reserve}$ was defined as the difference between resting and maximal HR from the baseline maximal exercise test. During the first 10 weeks of the intervention, training volume was gradually increased to 3 sessions of 40 minutes at 70% of $HR_{reserve}$ per week, and was then maintained for the final 14 weeks of the intervention. Subjects also added a lower-intensity, 45-60 minute exercise session during weeks 12-24. The aerobic exercise took place on a variety of exercise equipment including treadmills, as well as cycle, rowing, elliptical, and cross-country ski ergometers. Subjects were permitted to self-select the mode of exercise based on personal preference. For inclusion in the final analyses, subjects were required to have completed at least 75% of the scheduled exercise sessions.

Plasma VEGF protein levels

During baseline testing, subjects underwent an OGTT for evaluation of glucose metabolism. Prior to the onset of this test, fasting blood samples were collected in 10mL Vaccutainer® vials with 15% EDTA for the measurement of plasma VEGF levels. The samples were centrifuged at 3000 x g for 20 minutes and plasma was aliquotted into 2.0mL cryotubes and stored at -80ºC until measurement of plasma VEGF levels. The Human VEGF Quantikine ELISA Kit (R&D Systems, Minneapolis, MN) was used to measure plasma VEGF levels, following the manufacturer's instructions. After completion of the assay procedures, the optical density of each sample was measured using an Emax Microplate Reader (Molecular

Devices Corporation, Sunnyvale, CA). VEGF standards provided by the manufacturer were used, standard curves were generated according to the manufacturer's instructions, and data were fit using SOFTmax PRO v3.1 (Molecular Devices Corporation, Sunnyvale, CA). Each plasma sample was measured in duplicate and the average of those two measurements was used for statistical analyses.

As high plasma levels of soluble VEGF receptor-1 (sVEGFR-1, also known as sFlt-1) can interfere with plasma VEGF level measurement[69], the Human sVEGF R1 (Flt-1) Quantikine ELISA Kit (R&D Systems) was used to quantify sVEGFR-1 to determine if sVEGFR-1 interference exists. No sVEGFR-1 interference was detected, so all samples were included in the analysis of plasma VEGF levels.

Vo_{2max}

Before and after the exercise training intervention, subjects underwent a fixed-speed maximal treadmill exercise test to assess Vo_{2max}. After a brief warm-up, the test began at a workload corresponding to approximately 70% of the peak heart rate achieved on each subject's screening exercise test. Every 2 minutes the treadmill grade was increased by 2%. Vo_2 was measured continuously using a customized metabolic system (Rayfield Mixing Chamber, VMM Ventilatory Turbine, and Marquette Respiratory Mass Spectrometer). The test was terminated when a subject could no longer continue and standard physiological criteria were used to determine whether a true Vo_{2max} was achieved: respiratory exchange ratio greater than 1.15 or no further increase in Vo_2 (less than 150ml/min) with an increase in workload.

Electrocardiographic monitoring was continuous throughout the test and blood pressure monitoring was performed every 2 minutes throughout the test.

Body composition

Before and after the exercise intervention, percent body fat was measured using dual-energy X-ray absorptiometry (DPX-L; Lunar Corporation, Madison, WI) as previously described[99].

***VEGF* genotype and haplotype determination**

Genomic DNA was extracted from peripheral lymphocytes of whole blood samples using the PureGene® DNA extraction kit (Gentra Systems, Inc.).

The *VEGF* C936T SNP was genotyped by restriction fragment length polymorphism (RFLP) analysis[7]. A 208bp region surrounding the C936T SNP was amplified by polymerase chain reaction (PCR) with the forward primer 5'-ACA CCA TCA CCA TCG ACA GA-3' and reverse primer 5'-GCT CGG TGA TTT AGC AGC A-3'. The amplified DNA fragments were incubated with the restriction endonuclease *HpyCH4 III* overnight at 37°C and genotypes were visualized on a 2% agarose gel.

The VEGF C-7T SNP was genotyped by RFLP analysis[7]. A 420bp region surrounding the C-7T SNP was amplified by PCR with the forward primer 5'-GGC GTC GCA CTG AAA CTT TTC G-3' and reverse primer 5'-CCC AAG ACA GCA GAA AGT TCA TGG TTC C-3'. The amplified DNA fragments were incubated with the restriction endonuclease *BspE I* overnight at 37°C and genotypes were visualized on a 2% agarose gel.

The *VEGF* G-634C SNP was genotyped by RFLP analysis[7]. A 345bp region surrounding the G-634C SNP was amplified by PCR with the forward primer 5'-GTA GCA AGA GCT CCA GAG AGA AGT-3' and reverse primer 5'-TGG ACG AAA AGT TTC AGT GCG ACG-3'. The amplified DNA fragments were incubated with the restriction endonuclease *BsmF I* overnight at 65°C and genotypes were visualized on a 2% agarose gel.

The *VEGF* G-1154A SNP was genotyped by pyrosequencing[121]. A 193bp region surrounding the G-1154A SNP was amplified by PCR with the forward primer 5'-GTC GAG CTT CCC CTT CAT T-3' and reverse primer 5'-CCG CTA CCA GCC GAC TTT-3' with 5'-biotinylation. The internal pyrosequencing primer 5'-AGC CGC GTG TGG A-3' was used in the detection of the G-1154A genotype on a Pyrosequencing PSQ HS 96 light detection system (Pyrosequencing AB, Uppsala, Sweden) following instructions provided by the manufacturer.

The *VEGF* C-2578A SNP was genotyped by RFLP analysis[7]. A 317bp region (or 299bp region when the C-allele is present) surrounding the C-2578A SNP was amplified by PCR with the forward primer 5'-CTG ACT AGG TAA GCT CCC TGG A-3' and reverse primer 5'-AGC CCC CTT TTC CTC CAA CT-3'. The amplified DNA fragments were incubated with the restriction endonuclease *Bgl II* overnight at 37°C and genotypes were visualized on a 2% agarose gel.

VEGF promoter region haplotype (-2578/-1154/-634 SNPs, respectively) was determined from genotyping results where possible (i.e., when an individual was heterozygous for ≤1 of the 3 SNPs). For individuals heterozygous at ≥2 SNPs, *VEGF* promoter region haplotype was determined using a combination of allele-specific

PCR and RFLP. For example, haplotype determination for an individual heterozygous for the C-2578A and G-634C SNPs required: 1) Two PCR amplifications of the *VEGF* promoter region encompassing both SNPs, each with a reverse primer specific to either the -634 C-allele or G-allele and 2) digestion with the restriction endonuclease *Bgl II* to determine the -2578 allele that is on the same chromosome as either the -634 C-allele or G-allele. *VEGF* promoter region haplotypes and genotypes were confirmed by sequencing allele-specific PCR amplimers in a sample of 96 subjects.

DNA amplification for *VEGF* gene expression assays

VEGF promoter region haplotype was determined in DNA samples from human subjects participating in the *Studies of Human Genetic Sequence Variation* project (IRB# 01198). These subjects are a racially diverse group recruited to donate DNA samples for the purposes of screening for DNA sequence variation and for *in vitro* experimentation. DNA samples with AGG, AAG, CGG, and CGC haplotypes were selected and the *VEGF* promoter region from 5' position -3538 to -369 was amplified by PCR with the forward primer 5'-CCA GGT CAC AGC CAG GTT AT-3' and the reverse primer (with a mismatch incorporating the *Hind III* restriction sequence underlined): 5'-CCC AAG CTT TGG ACG AAA AGT TTC AGT GCG ACG-3' (Figure 1). The FastStart High Fidelity PCR System (Roche Applied Sciences, Indianapolis, IN) with a DNA polymerase with 3'-5' exonuclease ('proofreading') function was used to minimize amplification errors.

Vector preparation and cloning

The pGL3-Basic Reporter Vector (Promega, Madison, WI) was used for this study. This vector contains the firefly luciferase gene with an upstream multiple cloning site, but lacks eukaryotic enhancer and promoter sequences. This vector also contains the β-lactamase gene which confers ampicillin resistance to allow for selection of transformed bacteria.

The amplified *VEGF* promoter region DNA fragments containing the AGG and CGG haplotypes were purified by gel electrophoresis using the QIAquick Gel Extraction Kit (Qiagen, Inc., Valencia, CA), then both the reporter vector and the promoter region fragment were separately incubated with the *Hind III* and *Kpn I* restriction endonucleases in preparation for ligation. This took advantage of the *Hind III* and *Kpn I* recognition sequences in the pGL3-Basic multiple cloning site as well as the naturally occurring *Kpn I* recognition sequence upstream (5') of the -2578 SNP and the *Hind III* recognition sequence created by a mismatched amplification primer (see Figure 1). The promoter region fragment was then ligated into the pGL3-Basic Reporter Vector using T4 DNA Ligase (Invitrogen Corporation, Carlsbad, CA), following the manufacturer's instructions.

Recombinant plasmid vectors containing the AGG, CGG, CGC, and AAG haplotypes were constructed for this experiment (Figure 2). The AAG and CGC recombinant plasmid vectors were created from the AGG and CGG vectors, respectively, using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). This system utilizes two mutagenic primers and a DNA polymerase

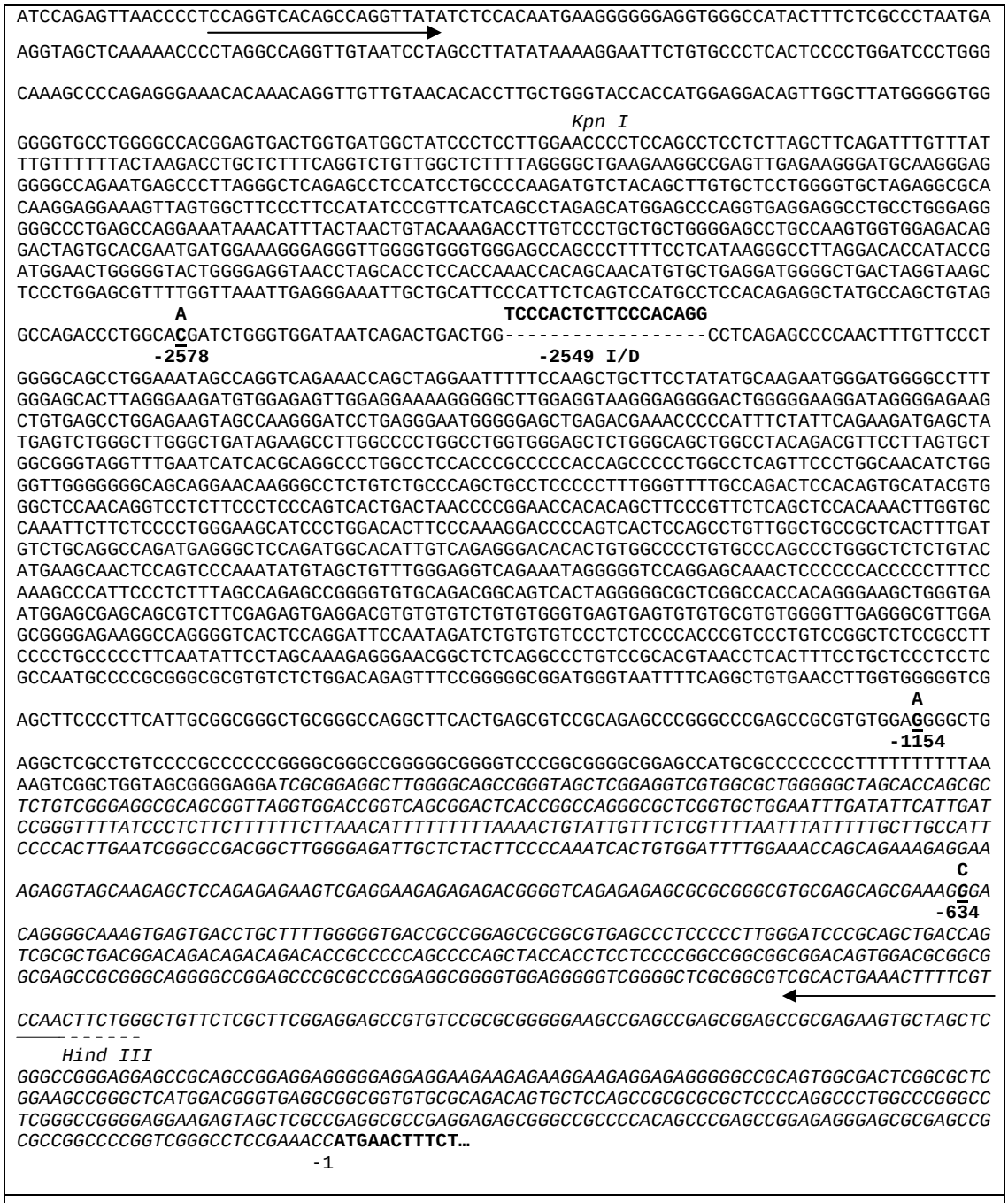

Figure 1. A diagram of the promoter region of the *VEGF* gene. The 5'UTR is shown in italicized font; translated nucleotides are shown in bold font. The binding sites of the PCR primers for the gene expression assays are designated by arrows (the dashed portion of one arrow designates the mismatch creating a *Hind III* recognition sequence). The *Kpn I* recognition site is underlined. Polymorphisms are designated by their position relative to the translation initiation codon (ATG).

with proofreading function to create the desired 'mutant' plasmid vectors while minimizing amplification errors. The recombinant plasmid vectors created by this method were screened and sequenced to confirm that no amplification errors or additional mutations were present in the DNA sequence.

The identified and sequence-confirmed recombinant plasmid vectors were subsequently transformed into chemically competent E. coli cells (Subcloning Efficiency DH5α, Invitrogen Corporation, Carlsbad, CA). After growth, the recombinant plasmid vectors were isolated from the E. coli cells using the ChargeSwitch® Plasmid ER Mini Kit (Invitrogen Corporation, Carlsbad, CA) to yield endotoxin-free recombinant plasmid vectors to be used for myoblast transfection.

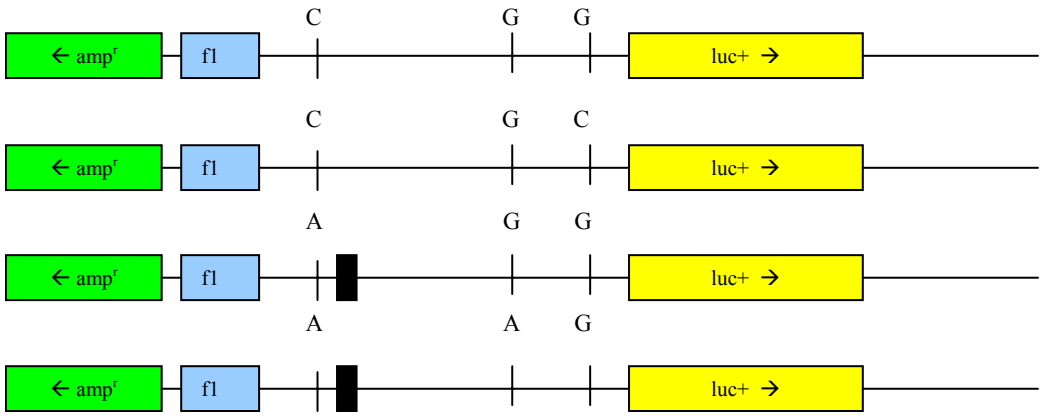

Figure 2. Diagram of experimental recombinant plasmid vectors indicating *VEGF* promoter region haplotype (-2578, -1154, and -634 alleles, respectively). The 18-bp insertion linked to the -2578 A-allele is indicated by a black box. Abbreviations: amp^r (ampicillin resistance gene), f1 (f1 bacterial origin of replication), and luc+ (firefly luciferase gene). Note: figure not drawn to scale.

Cell culture and transfection

Percutaneous biopsies of the vastus lateralis muscle were obtained from healthy, older (50-65 years of age) white women as part of the IRB-approved project *Race, Menopause, and Metabolism After Exercise and Diet* (University of Maryland at Baltimore IRB# M1174, P.I.: Alice S. Ryan, Ph.D.) and primary cultures of human myoblasts were generated in the laboratory of Dr. John McLenithan using a method similar to that described by Thompson et al.[144] Primary myoblast cultures were subcultured and thawed cell samples were split into no more than a 1:5 ratio. Myoblasts used for this experiment were no greater than passage 4. The myoblasts were cultured in Skeletal Muscle Basal Medium (Cambrex Corporation, East Rutherford, NJ) supplemented with 10% fetal bovine serum and incubated at 37°C with 5% CO_2. Cultured myoblasts were plated onto 24-well BioCoat Collagen I-coated culture plates (BD Biosciences Discovery Labware, Bedford, MA) and the cell culture medium was changed every other day to achieve 50-70% confluence on the day of transfection. For each experimental construct (i.e., for each different *VEGF* haplotype) 6 samples were transfected: 2 samples from each of 3 different primary cultures from different individuals.

Transfection of myoblasts was conducted with the Lipofectamine Plus Reagent (Invitrogen Corporation, Carlsbad, CA) following the manufacturer's instructions. In each well of the 24-well plates, transfection was performed with 2μl Lipofectamine, 4μl Plus reagent, 0.4μg of recombinant pGL3-Basic vector containing one *VEGF* promoter region haplotype, and 0.02μg of pRL-CMV vector (a vector with a cytomegalovirus (CMV) promoter-driven expression of Renilla luciferase used to

normalize for transfection efficiency). As an external control, transfection with 0.4μg of the pGL3-Basic vector (negative control without promoter or enhancer sequences) and 0.02μg of the pRL-CMV vector was also performed.

Cell culture incubation conditions

Two incubation conditions were used after transfection: normoxia (~20% ambient O_2) and hypoxia (~1% ambient O_2). All cells were initially incubated for 32 hours at 37°C with ~20% O_2 and 5% CO_2. For the normoxic condition, transfected cells were maintained in these conditions for an additional 16 hours. For the hypoxic condition, cell culture plates were placed in a 5310 Dessicator (Nalgene Labware, Rochester, NY). The dessicator was flushed with a low-oxygen gas mix (1% O_2, 5% CO_2, 94% N_2) for 20 minutes, then sealed and incubated at 37°C in a method similar to that described by Forsythe et al.[45] After 1 hour, the dessicator was again flushed with low-oxygen gas for 10 minutes to account for residual air that may have remained in the culture plates after the first flush. The dessicator was then sealed and incubated at 37°C for 15 hours.

Luciferase assays

The Dual-Luciferase Reporter Assay System (Promega, Madison, WI) was used to quantitate luciferase activity from transfected myoblasts. Growth medium was removed from transfected myoblasts 48 hours after transfection and the myoblasts were rinsed with phosphate buffer solution. The cells were then lysed and harvested using the lysis buffer provided by the manufacturer. The cell lysate (20μl) was mixed with Luciferase Assay Reagent II (100μl) and firefly luciferase signal

(from the experimental pGL3 vectors) was measured with a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA) programmed for a 2-second delay followed by a 10-second signal integration period. After measurement of the firefly luciferase signal, Stop & Glo Reagent (100µl) was mixed with samples and *Renilla* luciferase (pRL-CMV control) signal was measured in the same manner as above as an internal control for transfection efficiency. Each transfected sample was measured in duplicate to determine relative firefly luciferase activity corrected by *Renilla* luciferase activity, with the average of the two readings used for analyses. Relative luciferase activity is reported as arbitrary units using the luciferase activity from the AAG haplotype under normoxic conditions as the referent value.

Statistical procedures

Analysis of covariance (ANCOVA) and Student's t-tests were used to test for differences in plasma VEGF levels and Vo_{2max} among *VEGF* genotypes and haplotype groups, as well as for differences in ΔVo_{2max} among *VEGF* haplotype groups. Age, sex, and race were used as covariates in all Vo_{2max} analyses. Race and percent body fat were used as covariates in all analyses of plasma VEGF levels. As no race*genotype or race*haplotype interactions were observed, it was not necessary to compare means by race. For all individual genotype analyses, a type I error rate of $\alpha=0.05$ was selected and two-tailed probabilities are given. Additionally, for the individual genotype analyses, Student's t-tests were only performed when the overall genotype effect in the ANCOVA met the criteria for statistical significance ($P\leq0.05$). For all haplotype analyses, a type I error rate of $\alpha=0.05$ was selected and one-tailed probabilities are given because directional hypotheses for these analyses were

developed based on the results of the *VEGF* gene expression analysis. Pearson's correlation was used to test for correlation between plasma VEGF level and Vo_{2max}. Chi-square analysis (1 degree of freedom) was used to assess potential deviations of genotype distribution from Hardy Weinberg equilibrium. Linkage disequilibrium (r^2) among genotypes was estimated using Linkage Disequilibrium Analyzer v1.0[35].

The gene expression data were analyzed using a two-factor (2 x 4: $\%O_2$ x haplotype) factorial analysis of variance (ANOVA). The random portion of the mixed model was written to account for variation among individual myoblast donors and among individual myoblast donors within incubation chambers. Hypoxic induction of luciferase activity was analyzed separately using ANCOVA with individual myoblast donor as a covariate. Protected Student's t-tests were then used to test for differences among the 4 common *VEGF* promoter region haplotypes (AAG, AGG, CGG, and CGC). Data are reported as adjusted LS means ± SEM. A type I error rate of α=0.05 was selected for this analysis and two-tailed probabilities are reported.

RESULTS

Five individual *VEGF* polymorphisms were studied for association with Vo_{2max} before and after aerobic exercise training, and the *VEGF* -2578/-1154/-634 promoter region haplotype was studied for effects on *VEGF* gene expression and for association with Vo_{2max} before and after aerobic exercise training. All genotype and haplotype groups (except women with TT genotype at the C936T SNP) that underwent aerobic exercise training exhibited significant increases in Vo_{2max} ($P<0.01$). In general, subjects experienced modest, but statistically significant weight loss (~1.4kg, $P<0.05$), but no significant differences in weight loss were observed among genotype or haplotype groups. Although 46 subjects did not complete the exercise training regimen and were not included in the analyses of final Vo_{2max} or ΔVo_{2max}, this did not appear to influence our analyses of baseline Vo_{2max}, as no significant differences were observed in baseline Vo_{2max} between subjects who completed the exercise regimen and all subjects that completed baseline testing (Table 4a-e).

Table 1. Characteristics of a) all subjects used for the study of baseline Vo_{2max} and plasma VEGF levels, and b) subjects used for the study of final Vo_{2max} and ΔVo_{2max}.

a)

	Men	**Women**
Age (yrs)	58.6 ± 0.7 (n=79)	57.4 ± 0.5 (n=117)
Height (cm)	177.5 ± 0.8 (n=79)	162.9 ± 0.6 (n=117)
BMI (kg/m^2)	28.9 ± 0.5 (n=79)	29.0 ± 0.4 (n=117)
Baseline Weight (kg)	91.3 ± 1.6 (n=79)	76.9 ± 1.2 (n=117)
Baseline Vo_{2max} ($ml \cdot kg^{-1} \cdot min^{-1}$)	28.0 ± 0.5 (n=79)	22.0 ± 0.3 (n=117)
Plasma VEGF level ($pg \cdot ml^{-1}$)	18.8 ± 1.3 (n=40)	19.0 ± 1.4 (n=52)

Data presented as means ± SEM.

b)

	Men	Women
Age (yrs)	59.0 ± 0.8 (n=63)	57.7 ± 0.5 (n=87)
Height (cm)	177.9 ± 0.8 (n=63)	163.7 ± 0.7 (n=87)
BMI (kg/m^2)	28.5 ± 0.5 (n=63)	28.2 ± 0.5 (n=87)
Baseline Weight (kg)	90.3 ± 1.8 (n=63)	75.6 ± 1.4 (n=87)
Change in Weight (kg)	-1.8 ± 0.3 (n=63)	-1.1 ± 0.3 (n=87)
Baseline Vo_{2max} ($ml \cdot kg^{-1} \cdot min^{-1}$)	28.2 ± 0.6 (n=63)	22.4 ± 0.4 (n=87)
Final Vo_{2max} ($ml \cdot kg^{-1} \cdot min^{-1}$)	32.6 ± 0.7 (n=63)	25.4 ± 0.4 (n=87)

Data presented as means ± SEM.

Allele and haplotype frequencies

Allele frequencies for individual polymorphisms were calculated from 196 individuals with the exception of the -2578 and -1154 SNPs, which were calculated from 195 individuals as genotypes could not be determined for 1 subject (Table 2a). As haplotypes could not be determined for 5 subjects, haplotype frequencies were calculated from 191 individuals (Table 2b). Four common haplotypes (CGG, CGC, AGG, and AAG) comprised ~99% of all observed haplotypes, while four rare haplotypes (AAC, CAC, CAG, and AGC) comprised the remaining ~1%. With the exception of the G-634C polymorphism (P=0.04), no significant deviation from Hardy-Weinberg equilibrium expectations was observed (P=0.23-0.85). Differences in allele frequencies between race groups were observed with blacks having lower frequency of the rare allele than whites in all cases. Moderate, but statistically significant linkage disequilibrium (LD) was detected among the 4 variants upstream (5') of the *VEGF* translation initiation codon, as well as between the -1154 and 936 SNPs (Table 3).

Table 2. Allele frequencies for: a) individual *VEGF* polymorphisms and b) VEGF -2578/-1154/-634 haplotypes.

a)

	Whites (n=150*)			**Blacks** (n=46)	
	p	**q**		**p**	**q**
C-2578A	0.52 (C)	0.48 (A)		0.78 (C)	0.22 (A)
G-1154A	0.70 (G)	0.30 (A)		0.91 (G)	0.09 (A)
G-634C	0.72 (G)	0.28 (C)		0.81 (G)	0.19 (C)
C-7T	0.77 (C)	0.23 (T)		0.89 (C)	0.11 (T)
C936T	0.82 (C)	0.18 (T)		0.87 (C)	0.13 (T)

*n=149 for C-2578A and G-1154A SNPs.

b)

	-2578/-1154/-634 Haplotype				
	CGG	**CGC**	**AAG**	**AGG**	**Others**
Whites (n=145)	0.22	0.27	0.29	0.19	0.03
Blacks (n=46)	0.60	0.19	0.12	0.09	0.00

Table 3. Linkage disequilibrium values (r^2) for *VEGF* polymorphisms.

	-2578	**-1154**	**-634**	**-7**
-1154	0.37*			
-634	0.21*	0.10*		
-7	0.21*	0.02	0.03*	
936	0.02	0.05*	<0.01	<0.01

*Statistically significant LD, P<0.01. (n=195)

Vo_{2max} and individual *VEGF* polymorphisms

At baseline, the C-2578A SNP was not associated with Vo_{2max}; however, after aerobic exercise training, subjects with CC genotype exhibited lower Vo_{2max} (adjusted for baseline Vo_{2max}) compared to CA and AA genotypes (Table 4a). Similarly, the G-1154A SNP was not associated with baseline Vo_{2max}, but subjects of GG genotype exhibited lower Vo_{2max} (adjusted for baseline Vo_{2max}) after training compared to GA and AA genotypes (Table 4b). No significant differences in Vo_{2max} before or after training were observed among G-634C or C-7T genotypes (Tables 4c and 4d, respectively). The C936T SNP was not associated with baseline Vo_{2max}, but after aerobic exercise training, a sex*genotype interaction was detected (P=0.017) and data were analyzed by sex. In women, no differences in Vo_{2max} after aerobic exercise training (adjusted for baseline Vo_{2max}) were observed among genotype groups, but in men, the CC genotype at the C936T SNP was associated with a lower Vo_{2max} after aerobic exercise training (adjusted for baseline Vo_{2max}) compared to CT and TT genotypes (Table 4e).

Table 4. Vo_{2max} values for subjects by a) *VEGF* C-2578A genotype, b) *VEGF* G-1154A genotype, c) *VEGF* G-634C genotype, d) *VEGF* C-7T genotype, and e) *VEGF* C936T genotype.

a)

	C-2578A Genotype		
	AA	**CA**	**CC**
Baseline Vo_{2max} *all subjects* ($ml \cdot kg^{-1} \cdot min^{-1}$)	24.5 ± 0.69 n=33	25.0 ± 0.46 n=96	24.1 ± 0.45 n=66
Baseline Vo_{2max} *final subjects* ($ml \cdot kg^{-1} \cdot min^{-1}$)	24.9 ± 0.81 n=26	25.1 ± 0.52 n=79	24.5 ± 0.53 n=44
Final Vo_{2max} ($ml \cdot kg^{-1} \cdot min^{-1}$)	29.5 ± 0.67† n=26	29.0 ± 0.43‡ n=79	27.7 ± 0.44†‡ n=44
ΔVo_{2max} ($ml \cdot kg^{-1} \cdot min^{-1}$)	4.67 ± 0.67 n=26	4.13 ± 0.43 n=79	2.87 ± 0.44 n=44

Data are adjusted LS means ± SEM. All means are adjusted for age, sex, and race; final means are also adjusted for baseline Vo_{2max}. Overall genotype effect probabilities for baseline and final Vo_{2max} were P=0.36 and P=0.05, respectively. No significant differences in baseline Vo_{2max} were observed when all subjects and subjects completing exercise training (final subjects) were compared. Differences in ΔVo_{2max} among genotype groups were not tested. †‡Significant difference between genotype groups with like symbols: †P=0.039, ‡P=0.025.

b)

	G-1154A Genotype		
	AA	**GA**	**GG**
Baseline Vo_{2max} *all subjects* ($ml \cdot kg^{-1} \cdot min^{-1}$)	26.3 ± 1.1 n=11	25.4 ± 0.43 n=78	24.5 ± 0.36 n=106
Baseline Vo_{2max} *final subjects* ($ml \cdot kg^{-1} \cdot min^{-1}$)	26.0 ± 1.2 n=9	25.5 ± 0.46 n=64	25.1 ± 0.42 n=76
Final Vo_{2max} ($ml \cdot kg^{-1} \cdot min^{-1}$)	30.1 ± 0.99† n=9	29.0 ± 0.38‡ n=64	28.0 ± 0.35†‡ n=76
ΔVo_{2max} ($ml \cdot kg^{-1} \cdot min^{-1}$)	5.28 ± 0.98 n=9	4.20 ± 0.38 n=64	3.15 ± 0.35 n=76

Data are adjusted LS means ± SEM. All means are adjusted for age, sex, and race; final means are also adjusted for baseline Vo_{2max}. Overall genotype effect probabilities for baseline and final Vo_{2max} were P=0.15 and P=0.04, respectively. No significant differences in baseline Vo_{2max} were observed when all subjects and subjects completing exercise training (final subjects) were compared. Differences in ΔVo_{2max} among genotype groups were not tested. †‡Significant difference between genotype groups with like symbols: †P=0.044, ‡P=0.046.

c)

	G-634C Genotype		
	CC	**GC**	**GG**
Baseline Vo_{2max} *all subjects* ($ml \cdot kg^{-1} \cdot min^{-1}$)	25.5 ± 0.87 n=18	24.6 ± 0.51 n=63	24.3 ± 0.37 n=115
Baseline Vo_{2max} *final subjects* ($ml \cdot kg^{-1} \cdot min^{-1}$)	25.3 ± 0.95 n=15	25.3 ± 0.57 n=47	24.5 ± 0.43 n=88
Final Vo_{2max} ($ml \cdot kg^{-1} \cdot min^{-1}$)	28.7 ± 1.2 n=15	28.9 ± 0.74 n=47	28.2 ± 0.56 n=88
ΔVo_{2max} ($ml \cdot kg^{-1} \cdot min^{-1}$)	3.39 ± 0.80 n=15	3.59 ± 0.48 n=47	3.68 ± 0.36 n=88

Data are adjusted LS means ± SEM. All means are adjusted for age, sex, and race; final means are also adjusted for baseline Vo_{2max}. Overall genotype effect probabilities for baseline and final Vo_{2max} were P=0.41 and P=0.66, respectively. No significant differences in baseline Vo_{2max} were observed when all subjects and subjects completing exercise training (final subjects) were compared. Differences in ΔVo_{2max} among genotype groups were not tested.

d)

	C-7T Genotype		
	TT	**CT**	**CC**
Baseline Vo_{2max} *all subjects* ($ml \cdot kg^{-1} \cdot min^{-1}$)	22.6 ± 1.2 n=10	24.9 ± 0.53 n=56	24.5 ± 0.35 n=130
Baseline Vo_{2max} *final subjects* ($ml \cdot kg^{-1} \cdot min^{-1}$)	24.6 ± 1.6 n=5	24.7 ± 0.58 n=50	24.8 ± 0.41 n=95
Final Vo_{2max} ($ml \cdot kg^{-1} \cdot min^{-1}$)	28.2 ± 1.4 n=5	28.9 ± 0.49 n=50	28.3 ± 0.34 n=95
ΔVo_{2max} ($ml \cdot kg^{-1} \cdot min^{-1}$)	3.38 ± 1.4 n=5	4.07 ± 0.49 n=50	3.45 ± 0.34 n=95

Data are adjusted LS means ± SEM. All means are adjusted for age, sex, and race; final means are also adjusted for baseline Vo_{2max}. Overall genotype effect probabilities for baseline and final Vo_{2max} were P=0.20 and P=0.50, respectively. No significant differences in baseline Vo_{2max} were observed when all subjects and subjects completing exercise training (final subjects) were compared. Differences in ΔVo_{2max} among genotype groups were not tested.

e)

	C936T Genotype		
	TT	**CT**	**CC**
Men's Baseline Vo_{2max} *all subjects* ($ml \cdot kg^{-1} \cdot min^{-1}$)	29.4 ± 2.1 n=4	26.9± 0.96 n=22	26.8 ± 0.71 n=54
Women's Baseline Vo_{2max} *all subjects* ($ml \cdot kg^{-1} \cdot min^{-1}$)	23.8 ± 2.2 n=2	21.9 ± 0.62 n=31	21.6 ± 0.38 n=83
Men's Baseline Vo_{2max} *final subjects* ($ml \cdot kg^{-1} \cdot min^{-1}$)	29.8 ± 2.5 n=3	27.5 ± 1.1 n=20	27.3 ± 0.84 n=40
Women's Baseline Vo_{2max} *final subjects* ($ml \cdot kg^{-1} \cdot min^{-1}$)	23.8 ± 2.2 n=2	21.8 ± 0.71 n=23	22.0 ± 0.43 n=62
Men's Final Vo_{2max} ($ml \cdot kg^{-1} \cdot min^{-1}$)	36.2 ± 1.9 n=3	34.6 ± 0.83† n=20	32.7 ± 0.66† n=40
Women's Final Vo_{2max} ($ml \cdot kg^{-1} \cdot min^{-1}$)	23.2 ± 1.7 n=2	24.5 ± 0.54 n=23	25.0 ± 0.34 n=62
Men's ΔVo_{2max} ($ml \cdot kg^{-1} \cdot min^{-1}$)	7.98 ± 1.9 n=3	6.40 ± 0.82 n=20	4.51 ± 0.65 n=40
Women's ΔVo_{2max} ($ml \cdot kg^{-1} \cdot min^{-1}$)	0.86 ± 1.7 n=2	2.16 ± 0.54 n=23	2.69 ± 0.33 n=62

Data are adjusted LS means ± SEM. All means are adjusted for age and race; final means are also adjusted for baseline Vo_{2max}; ΔVo_{2max} means are adjusted for age and race. Overall genotype effect probabilities for baseline Vo_{2max} were P=0.50 and P=0.51 in men and women, respectively. No significant differences in baseline Vo_{2max} were observed when all subjects and subjects completing exercise training (final subjects) were compared. Overall genotype effect probabilities for final Vo_{2max} were P=0.05 and P=0.42 in men and women, respectively. Differences in ΔVo_{2max} among genotype groups were not tested. †Significant difference between genotype groups with like symbols: †P=0.038. In men, there was a tendency for final Vo_{2max} to be different among TT and CC genotype groups (P=0.0932).

***VEGF* gene expression**

Considering the associations of *VEGF* polymorphisms (particularly the -2578 and -1154 SNPs) with Vo_{2max} and the LD patterns among the *VEGF* promoter region polymorphisms, we conducted an experiment with the 4 common *VEGF* -2578/-1154/-634 haplotypes to assess potential effects on *VEGF* gene expression in

human myoblasts (characteristics of myoblast donors shown in Table 5). Under normoxic (~20% O_2) conditions, the AAG haplotype resulted in significantly lower luciferase activity than the CGG haplotype (P=0.02), and there was a tendency for the AGG and CGC haplotypes to result in lower luciferase activity than the CGG haplotype (Figure 3; P=0.0649 and P=0.0799, respectively). Under hypoxic (~1% O_2) conditions, the CGG haplotype resulted in significantly lower luciferase activity than the CGC haplotype (Figure 3; P=0.006) and the AGG haplotype resulted in significantly lower luciferase activity than the AAG and CGC haplotypes (Figure 3; P=0.013 and P=0.002, respectively). Likewise, the CGG haplotype resulted in lower hypoxic induction of luciferase activity (hypoxic luciferase activity relative to normoxic luciferase activity) than the CGC and AAG haplotypes (1.6-fold vs. 3.4-fold and 3.5-fold, P=0.0009 and P=0.0006, respectively; SEM=0.29), and the AGG haplotype resulted in significantly lower hypoxic induction of luciferase activity than the CGC and AAG haplotypes (2.1-fold vs. 3.4-fold and 3.5-fold; P=0.0094 and P=0.0065, respectively; SEM=0.29).

Table 5. Characteristics of subjects used for the study of *VEGF* gene expression.

	(n=3)
Age	58.3 ± 4.4
BMI ($kg \cdot m2^{-1}$)	28.9 ± 3.4
Vo_{2max} ($ml \cdot kg^{-1} \cdot min^{-1}$)	27.6 ± 7.4

Data presented as means ± SEM.

To confirm our results, this experiment was repeated with a smaller number of samples (n=4 for each haplotype). In the second experiment (data not shown), there

was a tendency for an effect of *VEGF* promoter region haplotype, with the same relationships observed between *VEGF* promoter region haplotypes and luciferase activity under both normoxic and hypoxic conditions that were observed in the first round of experiments (P=0.09 for haplotype main effect).

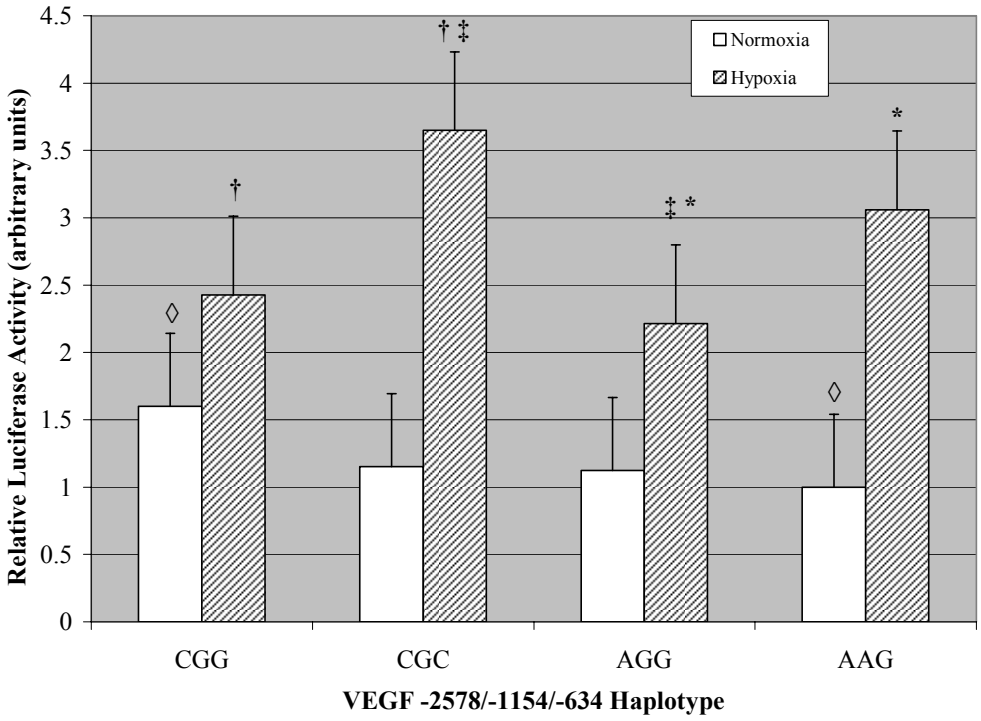

Figure 3. Relative luciferase activity values for *VEGF* -2578/-1154/-634 haplotypes. Data are presented as LS means with error bars representing the SEM. The main effect of haplotype was statistically significant (P=0.03) and significant increases in luciferase activity with hypoxia were observed in CGC and AAG haplotypes (P=0.006 and P=0.011, respectively). $^{*\lozenge\dagger\ddagger}$Significant difference between haplotypes with like symbols: *P=0.013, $^{\lozenge}$P=0.02, †P=0.006, ‡P=0.002.

***VEGF* promoter region haplotype, Vo_{2max}, and plasma VEGF levels**

Based on the results of the *VEGF* gene expression experiments under hypoxic conditions, subjects were grouped by *VEGF* -2578/-1154/-634 haplotype to test for

associations with Vo_{2max}. Subjects with only the AGG and/or CGG haplotypes comprised group 1, subjects with one copy of the AGG or CGG haplotype and one copy of the AAG or CGC haplotype comprised group 2, and subjects with only the AAG and/or CGC haplotypes comprised group 3 (Table 6). We hypothesized that Group 1 would exhibit lower Vo_{2max} before and after exercise training compared to Groups 2 and 3, and that Group 2 would exhibit lower Vo_{2max} before and after exercise training compared to Group 3. The results supported the hypothesis that Group 1 had lower Vo_{2max} before (P=0.013 and P=0.006) and after (P=0.006 and P=0.011) exercise training (not adjusted for baseline Vo_{2max}) than Groups 2 and 3, respectively, but there was only a tendency for Group 1 to have a lower ΔVo_{2max} with training compared to Groups 2 and 3 (P=0.073 and P=0.075, respectively). No significant differences were observed between Groups 2 and 3. All results are shown in Table 6.

Based on the results of the *VEGF* gene expression experiments under normoxic and hypoxic conditions, we next investigated whether plasma VEGF level was associated with *VEGF* -2578/-1154/-634 haplotype, as well as whether plasma VEGF level correlated with baseline Vo_{2max} or ΔVo_{2max} with aerobic exercise training. When subjects were grouped by *VEGF* promoter region haplotype according to the *VEGF* gene expression results under normoxic conditions (CGG vs. AGG, AAG, and CGC haplotypes), no association was observed between plasma VEGF level and *VEGF* -2578/-1154/-634 haplotype (Table 7a). Likewise, when subjects were grouped according to the *VEGF* gene expression results under hypoxic conditions, no association was observed between plasma VEGF level and *VEGF*

-2578/-1154/-634 haplotype (Table 7b). The results of the correlation analysis showed that there was no significant correlation between plasma VEGF level and baseline Vo_{2max} or ΔVo_{2max} with aerobic exercise training (r = -0.09, P=0.39 and r = 0.12, P=0.37, respectively).

Table 6. Vo_{2max} values for subjects grouped by *VEGF* -2578/-1154/-634 promoter region haplotype.

	***VEGF* -2578/-1154/-634 haplotype group**		
	1	**2**	**3**
Baseline Vo_{2max} *all subjects* ($ml \cdot kg^{-1} \cdot min^{-1}$)	$23.5 \pm 0.50^{\lozenge\dagger}$ n=53	$25.0 \pm 0.46^{\lozenge}$ n=86	$25.5 \pm 0.57^{\dagger}$ n=52
Baseline Vo_{2max} *final subjects* ($ml \cdot kg^{-1} \cdot min^{-1}$)	23.8 ± 0.58 n=37	25.4 ± 0.53 n=65	25.3 ± 0.62 n=45
Final Vo_{2max} ($ml \cdot kg^{-1} \cdot min^{-1}$)	$26.9 \pm 0.74^{\dagger\ddagger}$ n=37	$29.4 \pm 0.68^{\dagger}$ n=65	$29.4 \pm 0.80^{\ddagger}$ n=45
ΔVo_{2max} ($ml \cdot kg^{-1} \cdot min^{-1}$)	3.01 ± 0.49 n=37	3.97 ± 0.45 n=65	4.04 ± 0.53 n=45
Haplotypes included	***AGG/AGG*** ***AGG/CGG*** ***CGG/CGG***	***AGG****/AAG* ***CGG****/AAG* ***AGG****/CGC* ***CGG****/CGC*	*AAG/AAG* *AAG/CGC* *CGC/CGC*

Data are adjusted LS means ± SEM. All means are adjusted for age, sex, and race. $^{\lozenge\dagger\ddagger}$Significant difference between genotype groups with like symbols: $^{\lozenge}$P=0.013, †P=0.006, ‡P=0.011. There was a tendency for ΔVo_{2max} to be lower in group 1 compared to groups 2 and 3 (P=0.073 and P=0.075, respectively).

Table 7. Plasma VEGF levels for subjects grouped by *VEGF* -2578/-1154/-634 promoter region haplotype indicated by: a) gene expression results in normoxia, and b) gene expression results in hypoxia and Vo_{2max} haplotype analysis.

a)

	***VEGF* -2578/-1154/-634 haplotype**	
	A	**B**
Plasma VEGF level ($pg \cdot ml^{-1}$)	18.5 ± 2.1 n=22	17.8 ± 1.3 n=70
Haplotypes included	*CGG/CGG*	*All others*

Data are adjusted LS means ± SEM. All means are adjusted for race and percent body fat. (P=0.40 for haplotype group effect.)

b)

	***VEGF* -2578/-1154/-634 haplotype**		
	1	**2**	**3**
Plasma VEGF level ($pg \cdot ml^{-1}$)	17.3 ± 1.7 n=30	19.3 ± 1.5 n=47	15.9 ± 2.5 n=15
Haplotypes included	***AGG/AGG*** ***AGG/CGG*** ***CGG/CGG***	***AGG****/AAG* ***CGG****/AAG* ***AGG****/CGC* ***CGG****/CGC*	*AAG/AAG* *AAG/CGC* *CGC/CGC*

Data are adjusted LS means ± SEM. All means are adjusted for race and percent body fat. (P=0.21 for haplotype group effect.)

DISCUSSION

The principal findings of the present study are that: a) variation in the promoter region of the *VEGF* gene (i.e., *VEGF* -2578/-1154/-634 haplotype) impacted *VEGF* gene expression in human myoblasts under hypoxic conditions *in vitro*, b) the *VEGF* promoter region haplotypes impacting *VEGF* gene expression in human myoblasts had a concordant association with Vo_{2max} in older individuals before and after a standardized program of aerobic exercise training, and c) plasma VEGF level was not associated with *VEGF* promoter region haplotype, nor did plasma VEGF level correlate with baseline Vo_{2max} or ΔVo_{2max} with aerobic exercise training in older individuals. To our knowledge this is the first report that *VEGF* promoter region haplotype impacts *VEGF* gene expression in cultured human myoblasts and is associated with Vo_{2max}.

***VEGF* gene expression**

The present study investigated DNA sequence variation in the promoter region of the *VEGF* gene for effects on gene expression in cultured human myoblasts. Human myoblasts were chosen for investigation because they are the precursor to human myotubes and because the human myoblasts exhibited a relatively high level of VEGF mRNA expression in culture (unpublished data: S.J. Prior and J.C. McLenithan). The results of this investigation indicate that differences in *VEGF* gene expression exist as a function of *VEGF* promoter region haplotype in cultured human myoblasts.

In normoxic (~20% O_2) conditions, the CGG haplotype appeared to result in ~40% higher *VEGF* gene expression in cultured human myoblasts when compared to the CGC, AGG, and AAG haplotypes. However, the condition of ~20% O_2 in the tissue culture incubator may not be reflective of typical O_2 levels in skeletal muscle, and is considered by some to be supranormal[40]. This 20% O_2 level has been shown to elevate the Po_2 in cultured mouse skeletal muscle cells to ~40 Torr (approximately twice the normal level in mice and other mammals)[40]. Such an elevation of Po_2 in our cultured human myoblasts may have impacted the normal *VEGF* gene expression in these cells. Without assessing endogenous *VEGF* mRNA expression in individuals homozygous for these haplotypes, it cannot be known whether the results from human myoblasts *in vitro* actually reflect basal *VEGF* gene expression in skeletal muscle *in vivo*. Such an investigation was beyond the scope of the present study, but this question should be addressed in the future.

In hypoxic (~1% O_2) conditions, the AAG and CGC haplotypes resulted in ~43% higher *VEGF* gene expression in human myoblasts than did the CGG and AGG haplotypes. Eu et al.[40] have found that incubation in 1-2% O_2 results in a Po_2 of ~3.5 Torr in mouse skeletal muscle cells. As this O_2 level is likely to be representative of Po_2 in exercising skeletal muscle (2-4 Torr[116,147]) and is low enough to induce hypoxic activation of *VEGF* gene transcription[45,88,127,145], we think that this condition is a realistic simulation of Po_2 in exercising skeletal muscle.

In our experiments, the CGG and AGG haplotypes exhibited the lowest hypoxic induction (1.6-fold and 2.1-fold, respectively), and the CGC and AAG haplotypes exhibited the highest hypoxic induction of *VEGF* gene expression

(3.4-fold and 3.5-fold, respectively). Generally, this induction (1.6-fold to 3.5-fold) was lower than the increases in VEGF mRNA (2-fold to 6-fold[48,53,117,118]) after acute exercise *in vivo*, but this can likely be explained by 3 main factors. First, the 3'UTR sequence of the *VEGF* gene was not incorporated into the experimental reporter vector. So, while the hypoxic induction of *VEGF* gene transcription observed *in vivo* appears to have occurred in these reporter vectors, the 3'UTR-dependent hypoxic stabilization of endogenous VEGF mRNA[45,88,127,145] could not have occurred with these reporter vectors *in vitro*, likely resulting in less accumulation of reporter protein. Such stabilization of VEGF mRNA *in vivo* could partially explain the greater increases observed after acute exercise. Second, the regulation of *VEGF* gene transcription is a complex process involving numerous transcription factors and regulation pathways. Other exercise-related factors not included in the present experiments such as nitric oxide[36,49,72,94], tumor necrosis factor-α[36,104], and AMP-activated protein kinase[103] may directly or indirectly contribute to the larger VEGF mRNA increase observed *in vivo*. Third, it has been recently demonstrated that exercise-induced VEGF mRNA expression is lower in older women relative to their younger counterparts[31]. Although the mechanism for the effect of age on VEGF mRNA expression remains unclear, because our myoblasts were obtained from older subjects, age may have impacted the level of expression we observed.

Our results indicate a potential functional influence of the -1154 and -634 SNPs, such that the combination of G-alleles at these polymorphisms (i.e., A**GG** and C**GG** haplotypes) appears to result in lower *VEGF* gene expression in cultured human myoblasts relative to the other 2 haplotypes (A**AG** and C**GC**); the presence of the A-

or C-allele at the -2578 SNP (the first position in the haplotype) did not appear to affect the observed *VEGF* gene expression. At this time, however, a definitive statement cannot be made as none of these three polymorphisms occur within any specific transcription factor binding site identified to date[104] and the precise mechanism for *VEGF* promoter region haplotype effects on *VEGF* gene expression is still not known. As the hypoxia response element (HRE) in the VEGF promoter region (5' position -2012 to -2005) is upstream of these polymorphisms (but within the tested promoter region sequence) and requires interaction with an upstream activator protein-1 (AP-1: 5' position -2166 to -2160) and downstream activator protein-2α (AP-2: 5' position -1117 to -1110)[104], we speculate that the -1154 and/or -634 SNPs may affect these interactions in some manner. Also, as with any experiment performed *in vitro* using a reporter vector to quantitate gene expression, it is not clear whether the same results would have been observed if endogenous *VEGF* gene expression were measured in individuals with these haplotypes.

To our knowledge, this is the first investigation of *VEGF* promoter region haplotypes in human myoblasts, so direct comparisons to other reports are difficult or speculative; however, experiments with these *VEGF* promoter region haplotypes have been performed in other cell lines. Stevens et al.[141] investigated 3 *VEGF* promoter region haplotypes (AGG, AAG, and CGC) in MCF7 breast cancer cells using a similar luciferase reporter assay, finding that the AGG haplotype resulted in higher *VEGF* gene expression than the AAG or CGC haplotypes; however, it is important to note that this study intended to investigate additional polymorphisms within the *VEGF* promoter region, so these constructs differed at additional polymorphisms

(from that used in the present study), which makes comparing the present and former reports difficult. Lambrechts et al.[82] studied the same 3 *VEGF* promoter region haplotypes (AGG, AAG, and CGC) in GI-1 glioma cells, demonstrating that that the AAG and AGG haplotypes resulted in lower *VEGF* gene expression relative to the CGC haplotype in both normoxic and hypoxic (2% O_2) conditions. It should be stated, however, that the region from 5' position -2468 to -1177 (which contains the HRE) was absent in their reporter construct. While the results of the former and present studies are discordant, it is unclear whether this is due to differential regulation of *VEGF* gene transcription in different cell types (e.g., different transcription factors and/or signaling pathways), or due to differences in the experimental reporter constructs (i.e., different amounts of DNA sequence, the presence of additional DNA sequence variation, or the structure of the constructs themselves).

Vo_{2max}

The results of this investigation also demonstrate an association between *VEGF* gene sequence variation and Vo_{2max} in older individuals. When individual *VEGF* polymorphisms were analyzed, the -2578, -1154, and 936 SNPs were associated with Vo_{2max}, although the 936 SNP association was only observed in men. The mechanisms behind the association between the 936 SNP and Vo_{2max} are still unclear as the present study was not designed to evaluate them. Instead, we chose to focus our experiments on the *VEGF* promoter region polymorphisms previously shown to effect *VEGF* gene expression in different cell types. In order to better understand the associations of these individual *VEGF* promoter region

polymorphisms with Vo_{2max}, the *VEGF* gene expression experiments were designed and showed that *VEGF* promoter region haplotype has effects on *VEGF* gene expression in cultured human myoblasts. Based on the functional studies of these haplotypes under hypoxic conditions, subjects were grouped for analysis of association between *VEGF* promoter region haplotype and Vo_{2max}. We have chosen to group subjects based on the hypoxic gene expression results for two reasons: First, because the normoxic condition (~20% O_2) in the tissue culture incubator is not likely to reflect the O_2 tension in resting skeletal muscle; and second, we speculate that differences in Vo_{2max} as a function of *VEGF* promoter region haplotype would be the result of exercise- or hypoxia-induced *VEGF* gene and protein expression as opposed to basal *VEGF* gene and protein expression.

The results of the analyses of Vo_{2max} with these *VEGF* promoter region haplotype groups indicate that subjects with only CGG and/or AGG haplotypes (Group 1) exhibited significantly lower Vo_{2max} before and after exercise training than did subjects with at least one copy of the AAG or CGC haplotype (Groups 2 and 3), with a tendency for the same relationship in ΔVo_{2max}. These relationships are concordant with the results of the *VEGF* gene expression experiments, suggesting a functional effect of these polymorphisms in older individuals.

Interestingly, when analyzed by *VEGF* promoter region haplotype, our results indicate that the -1154 and -634 SNPs appear to be causing the observed associations, while the individual genotype analysis indicated that the -2578 and -1154 SNPs were associated with Vo_{2max} after aerobic exercise training, but the -634 SNP was not. We recognize that the genotype frequencies for the -634 SNP in our subjects differed

from Hardy Weinberg equilibrium expectations, but our genotype frequencies are similar to those from previous reports[82], so we do not believe that this significantly impacted our results. The association with the -2578 SNP and lack of association with the -634 SNP in the individual analyses may be attributable to LD patterns in the *VEGF* promoter region and to the frequency of haplotypes in those genotype groups. For example, in the individual genotype analysis of final Vo_{2max}, within the AA genotype group at the -2578 SNP, the higher frequency of the A**AG** haplotype relative to the A**GG** haplotype may have resulted in a higher Vo_{2max} estimate, while within the CC genotype group the higher frequency of the C**GG** haplotype relative to the C**GC** haplotype may have resulted in a lower Vo_{2max} estimate. We share the view of others[33] that, when possible, polymorphisms should be studied not in isolation, but by haplotype (combinations of linked polymorphisms) to better reflect their function in their genetic context. Therefore, we feel that our *VEGF* promoter region haplotype results are a better reflection of the impacts of these polymorphisms than the results of the individual SNP analyses.

The most likely mechanism to explain the association between *VEGF* promoter region haplotype, *VEGF* gene expression, and Vo_{2max} is that differences in *VEGF* gene expression due to *VEGF* promoter region haplotype translate into differences in VEGF protein expression and localized secretion by exercising skeletal muscle, with downstream effects on skeletal muscle capillarity, and oxygen extraction by exercising skeletal muscle. However, as the current study did not assess VEGF protein expression in skeletal muscle *in vivo*, nor were skeletal muscle capillarity data available for these subjects, these issues remain to be addressed.

Plasma VEGF level

Analyses of plasma VEGF level as a function of *VEGF* promoter region haplotype and in relation to Vo_{2max} were conducted to determine whether plasma VEGF level can be used as an indicator of *VEGF* gene expression in skeletal muscle or as a predictor of baseline Vo_{2max} or ΔVo_{2max} with aerobic exercise training. Our results indicate neither an association between *VEGF* haplotype and plasma VEGF level, nor a correlation between plasma VEGF level and Vo_{2max}.

Our results are in partial disagreement with those of Lambrechts et al.[82] Their group found that *VEGF* promoter region haplotype was associated with plasma VEGF levels in a sample of European patients with ALS. The ALS patients with AAG and/or AGG haplotypes exhibited lower plasma VEGF levels than all other haplotypes combined, but plasma VEGF levels were markedly lower in the group of ALS patients than in their spouses, and the haplotype associations observed in ALS patients were not statistically significant in the group of healthy spouses[82]. As the goal of this project was to assess plasma VEGF level in relation to *VEGF* gene expression in skeletal muscle, subjects were not grouped according to the results of Lambrechts et al.[82] in any analysis. Additionally, Renner et al.[114] identified an association between plasma VEGF level and the C936T SNP, such that carriers of the T-allele (TT genotype) exhibited lower plasma VEGF levels compared to subjects of CC genotype in a group of healthy young men. Again, as it was not the goal of the current project to associate individual *VEGF* polymorphisms with plasma VEGF levels and the effects of the C936T SNP on *VEGF* gene expression in skeletal muscle are unknown, no analysis was performed to confirm the results of Renner et al.[114]

Hiscock et al.[59] and Kraus et al.[81] have reported increases in plasma VEGF after an acute bout of aerobic exercise, indicating that skeletal muscle does secrete VEGF protein into the circulation, but the relevance of that protein is still unresolved. While our results indicate that plasma VEGF level under resting conditions is not reflective of *VEGF* gene expression in human myoblasts, whether or not plasma VEGF level after acute exercise is reflective of *VEGF* gene expression in skeletal muscle or predictive of Vo_{2max} remains to be seen. The measures of plasma VEGF level and skeletal muscle VEGF protein level after acute exercise needed to address this question were not available for study in this project.

Summary

The present report shows significant associations between *VEGF* promoter region haplotype and Vo_{2max} before and after exercise training that are consistent with *VEGF* gene expression differences found for those *VEGF* promoter region haplotypes in cultured human myoblasts. While factors other than hypoxia and VEGF certainly contribute to Vo_{2max} before and after aerobic exercise training, these *VEGF* haplotype groups were associated with ~8-10% differences in Vo_{2max} both before and after 24 weeks of aerobic exercise training. While these results have potential implications for aerobic exercise training and the risk of morbidity/mortality that is associated with cardiorespiratory fitness (i.e., Vo_{2max}), our findings may also provide direction in understanding the function of the *VEGF* promoter region in different tissues under conditions of hypoxia, as well as prove relevant in the study of conditions such as obesity, cancer, coronary artery disease, and peripheral artery disease.

CONCLUSIONS

Hypothesis 1: *Vo_{2max} before and after exercise training differs among subjects with different genotypes at the -2578, -1154, -634, -7 and 936 VEGF gene polymorphisms.*

No association was observed between individual *VEGF* polymorphisms and Vo_{2max} before aerobic exercise training. The -2578, -1154, and 936 VEGF gene polymorphisms were associated with Vo_{2max} after aerobic exercise training, although the association with the 936 polymorphism was only observed in men.

Hypothesis 2: *VEGF gene expression (as quantified by luciferase activity) under a) normoxic and b) hypoxic conditions will differ among the 4 common VEGF promoter region haplotypes: AGG, AAG, CGG, and CGC (-2578, -1154, and -634 polymorphisms, respectively).*

VEGF gene expression differed among *VEGF* promoter region haplotypes in cultured human myoblasts under normoxic (~20% O_2) conditions, such that the CGG haplotype resulted in higher *VEGF* gene expression than the other 3 common haplotypes.

VEGF gene expression also differed among *VEGF* promoter region haplotypes in cultured human myoblasts under hypoxic (~1% O_2) conditions, with the AGG and CGG haplotypes having lower *VEGF* gene expression than the AAG and CGC haplotypes.

Hypothesis 3: *Based on the VEGF gene expression results under hypoxic conditions, Vo_{2max} before and after aerobic exercise training and ΔVo_{2max} with training will be lower in subjects with the AGG and/or CGG VEGF promoter region haplotypes than in subjects with the AAG and/or CGC haplotypes.*

In healthy older individuals, Vo_{2max} before and after aerobic exercise training was lower in subjects with only the AGG and/or CGG haplotypes compared to all other common haplotype groups. Likewise, there was a tendency for ΔVo_{2max} with aerobic exercise training to be lower in subjects with only the AGG and/or CGG haplotypes compared to all other common haplotype groups.

Hypothesis 4: *Based on the VEGF gene expression results under normoxic conditions, plasma VEGF level before aerobic exercise training will be higher in subjects with only the CGG VEGF promoter region haplotype than in subjects with the AAG, AGG and/or CGC haplotypes.*

Contrary to our hypothesis, subjects with the CGG haplotype did not have higher plasma VEGF levels than subjects with AAG, AGG, or CGC haplotype. In an additional analysis based on the *VEGF* gene expression results under hypoxic conditions, subjects with the AGG and/or CGG haplotypes did not have lower plasma VEGF levels than subjects with AAG and/or CGC haplotypes.

REVIEW OF LITERATURE

Maximal oxygen consumption

Morbidity, mortality, and Vo_{2max}: Maximal oxygen consumption (Vo_{2max}) is inversely associated with cardiovascular and all-cause mortality[28,42,75,84,98,151]. This relationship has been well demonstrated in men[12,28,75,84,151] and women[12,42,51], as well as in individuals who exhibit hypertension[10,28], hyperlipidemia[10], metabolic syndrome[75], obesity[151], and those who smoke[10]. Additionally, changes in cardiorespiratory fitness over time result in lower risk of mortality, whereas individuals who improve physical fitness are less likely to die from cardiovascular disease mortality and all-cause mortality[11,38].

Sex, race, and age differences in Vo_{2max}: Vo_{2max} has been shown to differ among groups of sedentary men and women, with women typically exhibiting lower Vo_{2max}[79,138,139] that may be attributed to smaller heart size[66], lower stroke volume[139], and lower hemoglobin levels[32]. However, the relative response of Vo_{2max} to aerobic exercise training (% increase in Vo_{2max}), appears to be similar among men and women[79,138]. Differences in Vo_{2max} have also been observed among different racial groups, particularly when comparing blacks and whites. In sedentary individuals, black subjects typically exhibit lower Vo_{2max} than their white counterparts[4,37,64,138] which may be attributable to differences in hemoglobin levels[4], differences in fat mass[138], and/or the degree of sedentary lifestyle[4]. The relative response of Vo_{2max} to aerobic exercise training appears similar among races[138]. Additionally, an inverse relationship exists between age and Vo_{2max} in the sedentary state, such that in

sedentary individuals, Vo_{2max} typically declines ~5-10% per decade[26,54,120,138,139]. As with sex and race, no significant differences appear to exist in the relative response of Vo_{2max} to aerobic exercise training among subjects of different age[54,55,79,138].

Vo_{2max} and aerobic exercise training: Vo_{2max} is responsive to aerobic exercise training, such that Vo_{2max} typically increases 15-30% in humans after 3-9 months of training[21,55,122]. Part of this increase in Vo_{2max} is attributed to central cardiovascular adaptations (i.e., increased cardiac output), with the remainder being attributed to increased oxygen extraction by the working muscle[122,125]. Such an increase in oxygen extraction may be achieved through increases in the capillarity of skeletal muscle (i.e., angiogenesis).

Angiogenesis

The process of angiogenesis: Angiogenesis is the formation of blood vessels from pre-existing vessels and vascular endothelial cells[119,123]. The formation of new capillaries is a critical phenomenon during pathological (e.g., cancer) and physiological conditions (e.g., aerobic exercise) to enhance the blood:tissue exchange capacity for oxygen delivery, metabolite removal, etc. Angiogenesis is a complex process involving a number of molecules acting to stimulate growth, direct migration, and stabilize new vessels. Angiogenesis typically occurs in two manners: intussusception (the splitting of one vessel into two) and sprouting of new vessels[24,119].

A key step in the angiogenic process is the activation of endothelial cells. The most potent activator of endothelial cells appears to be vascular endothelial growth

factor (VEGF). VEGF is an endothelial cell-specific mitogen that activates endothelial cells by binding to one of two different receptors on the surface of endothelial cells: VEGF receptor 1 (VEGFR1 or Flt-1) and VEGF receptor 2 (VEGFR2 or Flk-1)[44]. As activated endothelial cells begin to proliferate, the capillary basement membrane and extracellular matrix (ECM) begin to degrade. Degradation occurs in part due to the action of matrix metalloproteinases (MMPs) as VEGF can stimulate endothelial cells to produce pro-MMPs[83,148] which are converted to functional MMPs by the urokinase[56] and tissue plasminogen activators uPA and tPA (both upregulated by VEGF signaling)[68,92,93]. Furthermore, angiopoietin 2 (Ang2) is upregulated, resulting in the destabilization of local vasculature[109]. This degradation and destabilization allows new endothelial cells to migrate into the area and begin to form a new capillary.

The initiation and progression of capillary formation is guided by molecules that adhere to the surface of endothelial cells. Examples of these surface adhesion molecules include integrins (e.g., $alpha_v$-$beta_3$) and ECM-bound VEGF isoforms[108]. These molecules act to attract endothelial cells in an effort to regulate proper tube growth while minimizing unnecessary growth. As the newly formed capillary must rejoin another existing capillary at its terminus, the coordination of tube formation by these molecules is critical to achieve functional increases in vasculature[108].

Angiogenesis in response to exercise training: Aerobic exercise has been identified as a powerful angiogenic stimulus as several studies in the last 3 decades have shown increases in skeletal muscle capillarity after aerobic exercise training[1,21,101], with increases of ~34% in as little as 3 months of training[1]. There are

at least three potential mechanisms/stimuli for the increase in skeletal muscle capillarity with aerobic exercise training: hemodynamic stimuli, muscle contraction, and metabolic stimuli.

Hemodynamic stimuli such as changes in vessel wall tension, shear stress, pressure and flow may mediate angiogenesis by activating molecules such as nitric oxide or through direct effects on endothelial cells[36,49,61,72,94]. Muscle contraction may mediate the angiogenic process by promoting degradation of the ECM or by physically aiding the migration of endothelial cells and coordination of capillary formation[65,108,119,123,154]. Lastly, metabolic stimuli such as low oxygen tension[23,52,67,95,135] have significant effects on angiogenesis through the regulation of expression of growth factors as well as the expression of other factors and receptors[108].

Angiogenesis and Vo_{2max}: An important determinant of Vo_{2max} is skeletal muscle capillarity as these variables directly correlate[50] and one contributing mechanism to the increase in Vo_{2max} with aerobic exercise training is an increased skeletal muscle capillary network achieved by angiogenesis[21]. Increases in oxygen extraction by trained skeletal muscle have been demonstrated in both humans[115] and in other animals[58,153]. Increases in skeletal muscle capillarity (and thus capillary volume in a given volume of skeletal muscle) observed with exercise training can contribute to this increase in oxygen extraction by increasing the capillary surface area for diffusion, decreasing the average O_2 diffusion path length of skeletal muscle, and increasing red blood cell transit time through skeletal muscle[109].

Angiogenesis and disease: Angiogenesis not only plays a role in physiological processes in healthy individuals, but can play a role in adverse and pathological conditions. This role is prominent in occlusive arterial diseases (i.e., coronary artery disease (CAD) and peripheral artery disease (PAD)) where blood flow to localized tissues is impaired. Remodeling of existing vasculature and genesis of new vasculature have the ability to mediate physiological responses to CAD and PAD by increasing collateral circulation and oxygen supply[89]. Previous experiments have shown that administration of exogenous VEGF or adenovirus-mediated *VEGF* gene transfer can increase skeletal muscle capillarization and blood flow in a rat model[133]. Also, Couffinhal, et al. have demonstrated that an increase in endogenous VEGF protein expression induced by hindlimb ischemia in a mouse model was sufficient to increase capillary density and blood flow in skeletal muscle compared to controls[29]. Furthermore, aerobic exercise training has been shown to result in increased capillarization in the myocardium[62,152] and has been demonstrated as a valuable treatment for PAD[39].

Not only can angiogenesis play a role in muscle, but the effects of angiogenesis are of great relevance to conditions such as cancer and obesity. It has been demonstrated that tumor growth is promoted by angiogenesis[111] and several studies have demonstrated that the inhibition of angiogenesis prevents tumor growth[15,70,87,143]. Likewise, as adipose tissue is highly vascularized and has angiogenic properties[30,137], it has been hypothesized that adipose tissue mass can be regulated through the vasculature. At least two reports have demonstrated that that adipose tissue mass in obese (*ob/ob*) mice can be decreased significantly by the

inhibition of angiogenesis[80,124]. Taking these results into consideration, it is plausible that inter-individual variation in angiogenesis due to genetic factors could play a role in obesity and tumor development.

Vascular Endothelial Growth Factor

The VEGF gene and protein: VEGF protein is encoded by the *VEGF* gene (located on chromosome 6p12) which contains 8 exons separated by 7 introns. As a result of alternative splicing and an alternate translation initiation codon (539bp upstream of the canonical ATG codon), the *VEGF* gene is capable of generating 6 polypeptides, 3 of which are preferentially expressed in non-placental tissues ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$)[16]. $VEGF_{121}$ is non-heparin-binding and is freely diffusible; $VEGF_{189}$ is highly sequestered in the ECM[60], and $VEGF_{165}$ has intermediate properties that allow it to be bound to the ECM and diffusible[106]. The ECM-bound isoforms can be cleaved to produce an additional diffusible isoform ($VEGF_{110}$) to yield three diffusible VEGF isoforms ($VEGF_{110}$, $VEGF_{121}$, and $VEGF_{165}$)[60]. Although $VEGF_{165}$ is considered to be the most potent isoform because its affinity for the neuropilin receptor (NRP1) enhances its binding to the VEGFR2 receptor[44], these isoforms are otherwise considered to be similar in action[16].

VEGF and angiogenesis: Several studies have revealed a major regulator of angiogenesis to be vascular endothelial growth factor (VEGF)[43,131,132]. VEGF is an endothelial cell proliferation[85,119,123,128], mobilization[5,73,97], and migration factor[65,119,123] that is a potent stimulator of angiogenesis[44,86,140]. The diffusible isoforms of VEGF all bind to receptors on the abluminal surface of endothelial cells

promoting proliferation[44,132] and Hutchings et al.[65] have shown that endothelial cells can directly adhere to and migrate on ECM-bound VEGF.

VEGF gene and protein expression: VEGF is expressed in numerous human tissues including skeletal muscle. Numerous physiological stimuli have been shown to affect VEGF expression in skeletal muscle including metabolic processes, blood flow, and muscle contraction[108]. These stimuli appear to affect VEGF mRNA and protein expression through several factors and signaling pathways including: estrogen[104], nitric oxide[36,49,72,94], tumor necrosis factor-α[36,104], glucose concentration[36] AMP-activated protein kinase[103], forkhead transcription factor foxo1[46], cyclooxygenase[72], and hypoxia-inducible factor-1 (HIF1)[23,52,67,95,104,135].

VEGF, aerobic exercise and angiogenesis: Recent research has indicated that VEGF is indeed involved in the angiogenic response to aerobic exercise[20,53,89,101,118]. Several studies have confirmed that aerobic exercise increases VEGF mRNA, citing 2-fold to 6-fold increases in both animal[9,20,89,101] and human studies[48,53,117,118].

In the context of aerobic exercise, hypoxia is the best studied regulator of *VEGF* expression to date. Aerobic exercise has been shown to result in a local hypoxic condition in exercising skeletal muscle as Po_2 can reach levels as low as 2-4 Torr[116,147], a level low enough to induce hypoxic upregulation of *VEGF* gene expression and mRNA stabilization[45,88,127,145]. Hypoxic regulation is mediated through HIF-1 and its oxygen-sensing subunit HIF-1α, evidenced by several studies demonstrating that hypoxic or ischemic conditions result in increased VEGF mRNA and/or protein levels in biological tissues [23,52,67,95,135].

Studies performed *in vitro* using adenoviral vector encoding HIF-1α[88] and dibenzoylmethane (a compound that stabilizes HIF-1α)[90] have demonstrated a substantial increase in VEGF mRNA and protein, as the stable HIF-1α allowed for increased formation of functional HIF-1 protein. Stein et al.[140] and Ikeda et al.[67] have shown that hypoxia also increases VEGF mRNA half-life from ~40min in normoxia to ~2 hours in hypoxic conditions. To confirm the effects of hypoxia on endothelial cells, Gu et al.[52] used a DNA synthesis assay to demonstrate that human umbilical vein endothelial cells (HUVECs) treated with hypoxia-conditioned media experienced DNA synthesis at a rate two-fold greater than HUVECs in normal media, signaling greater mitogenic activity. When these same assays were run in the presence of anti-human VEGF antibody, the rate of DNA synthesis decreased in a dose-dependent fashion, indicating that hypoxia-mediated endothelial cell proliferation is primarily due to the action of VEGF protein[52]. Considering these studies and the role of VEGF, there is a considerable body of evidence suggesting that VEGF plays an integral role in the angiogenic response to aerobic exercise training.

Circulating VEGF protein level: As measured by ELISA, plasma VEGF level reportedly ranges from <9-150pg/ml[69]. Serum levels of VEGF are typically ~6-fold greater than plasma levels as VEGF is released from platelets and other blood cells during clotting[69]. Although serum and plasma VEGF levels correlate moderately well[81], serum measures are considered to be more reflective of blood platelet counts than of synthesis by peripheral tissues[69].

Although the prognostic value of circulating VEGF protein levels is not well established, there have been several reports that circulating VEGF protein levels are elevated in PAD[14], CAD[14], hypertension[8], hyperlipidemia[13], congestive heart failure[27], and obesity[96]. The report on obesity from Miyazawa-Hoshimoto et al.[96] further identified visceral fat area as significant determinant of serum VEGF level.

To date, few reports of the relationship between plasma VEGF level and exercise-related traits exist. Hiscock et al.[59] found that VEGF protein was increased after 3 hours of knee-extension ergometry in venous plasma, but not in arterial plasma. Kraus et al.[81] found that VEGF protein in venous plasma is increased from 0-2 hours after acute, systemic aerobic exercise, but only in endurance-trained individuals. These reports provide evidence that skeletal muscle does secrete VEGF protein into the circulation, but the relevance, abundance, and localization of that protein is still unresolved.

Lastly, although plasma VEGF level correlates well with the efficacy of treatments directed at angiogenic targets in clinical trials[136] and plasma VEGF levels are viewed as markers of angiogenic activity[76], the usefulness of plasma VEGF levels as predictors of skeletal muscle capillarity, Vo_{2max}, and the response of those variables to exercise training is still unknown.

Variability and heritability of Vo_{2max}, vasculature, and VEGF

Vo_{2max}: Significant variability is observed in Vo_{2max}, even among humans of the same age, sex, and race[19]. Furthermore, substantial inter-individual variability exists in the response of Vo_{2max} to aerobic exercise training[130]. While some

proportion of this variability can be attributed to non-genetic factors (e.g., habitual physical activity), there appears to be a significant contribution of genetic or familial factors. Twin studies have revealed correlations of Vo_{2max} between siblings ranging from 0.71-0.95 in monozygotic twins and 0.36-0.51 in dizygotic twins[18,77,78]. Additional research has estimated the heritability of Vo_{2max} to be as high as 59%[17,41] and the heritability of the response of Vo_{2max} to aerobic exercise training (ΔVo_{2max}) to be as high as 47%, though it is recognized that non-genetic familial influences contribute to these estimates[17].

Skeletal muscle capillarity: In humans, significant variability is also observed in the skeletal muscle capillary network, where capillary to fiber ratio in overall skeletal muscle has been shown to range from 0.81-1.97 cap/fiber among groups of similar individuals[21,105,107,134]. Additional variability in the capillarity of type I and type II skeletal muscle has also been observed[107], but this varies depending on the metric used (i.e., capillary density, capillary contacts per fiber, or fiber area per capillary) and may be dependent on differences in muscle fiber size[34].

The variability observed in overall skeletal muscle capillarity may contribute to Vo_{2max} through differences in local oxygen availability. While the genetic contribution to skeletal muscle capillarity has yet to be defined, investigators have argued that differences in the vasculature can be attributed to both environmental factors (e.g. aerobic exercise training), and genetic factors[21,114]. In fact, preliminary data from our laboratory indicates association between skeletal muscle capillarity and SNPs in the *VEGF* gene, suggesting a genetic component to skeletal muscle capillarity[110].

Plasma VEGF levels: Variability has also been observed in plasma VEGF levels among healthy subjects[91,114,150]. To date, the heritability of plasma VEGF levels has not been well studied; nonetheless, at least four reports have demonstrated associations between polymorphisms in the *VEGF* gene and plasma VEGF levels[82,114] or VEGF production *in vitro*[129,149], providing evidence of a genetic contribution.

VEGF gene expression: Variability in *VEGF* gene expression among individuals has been noted in several studies of human skeletal muscle, particularly after an acute aerobic exercise stimulus, where 2-fold to 6-fold increases in VEGF mRNA have been reported[48,53,117,118]. Additional evidence of variability in *VEGF* gene expression comes from a study of hypoxic induction of VEGF mRNA where Schultz et al.[126] demonstrated a range of ~1-fold to 7-fold induction of VEGF mRNA expression in monocytes derived from 51 individuals with CAD. Interestingly, monocytes derived from patients deemed to have no collateral circulation formation exhibited the lowest hypoxic induction of VEGF mRNA (1.9-fold), while monocytes derived from patients with 1+ or 2+ collateral circulation development exhibited higher VEGF mRNA induction (2.8-fold and 3.4-fold, respectively)[126]. This serves to demonstrate the variability of *VEGF* gene expression and the potential functional implications of that variability on the vasculature. The genetic contribution to *VEGF* gene expression has not been well studied, but at least two recent reports have demonstrated that polymorphisms within the *VEGF* gene affect *VEGF* gene expression in specific cell types *in vitro*[82,141], indicating a genetic contribution to *VEGF* gene expression.

***VEGF* as candidate gene for Vo_{2max}**

The investigation of genetic factors underlying physical traits has progressed through the study of so-called 'candidate genes' that are likely to play a role in a given physiological process based on their known function. *VEGF* is an important candidate gene for angiogenesis because the DNA sequence of the gene is polymorphic and VEGF has been identified as a potent angiogenic factor under both physiological and pathological conditions[86,140].

The DNA sequence in the promoter region of human genes is well known to bind enhancers and other regulators of DNA transcription and the 5' untranslated region (5'UTR) is known to regulate VEGF expression at the posttranscriptional level[63]. Likewise, the 3' untranslated region (3'UTR) of the *VEGF* gene has the ability to regulate translation as factors bind the 3'UTR to stabilize VEGF mRNA[88,102]. Therefore, variation in these regions of the *VEGF* gene may regulate *VEGF* gene transcription and translation, with potential effects on VEGF protein expression and 'downstream' traits such as Vo_{2max}.

Identified polymorphisms in the VEGF gene: Numerous polymorphisms have been identified in the *VEGF* gene and 3kb of its upstream (5') promoter region[22,114,141,149]. The majority of these polymorphisms occur at frequencies too low for study in this project (rare allele frequencies < 0.02)[141,149] and therefore, have little likely clinical significance. Of the remaining 7 common polymorphisms (rare allele frequency >0.13)[22,141,149], 6 have been previously investigated for association with and/or effects on *VEGF* gene and/or protein expression in select human tissues.

Investigations of common VEGF gene polymorphisms: Among the *VEGF* gene polymorphisms that have been investigated, the C-1498T (located in the promoter region, 1498 bp upstream of the translation initiation codon)[141,149] and G1612A (located in the 3'UTR, 913bp downstream of the translation termination codon)[114] single nucleotide polymorphisms (SNPs) have been studied in relation to *VEGF* gene and/or protein expression with no significant associations reported.

The C936T SNP is located in the 3'UTR, 37bp downstream of the coding region in exon 8[114]. Renner et al.[114] reported that carriers of the T-allele (CT and TT genotypes) at position 936 exhibited significantly lower plasma VEGF levels than did CC homozygotes in a group of 23 healthy young men.

The G-634C SNP is located 634bp upstream (5') of the canonical translation initiation codon, within the 5'UTR. Lambrechts et al.[82] have shown that L-VEGF (the precursor to $VEGF_{206}$) protein expression in C6 glioma cells is 20% lower with the -634 G-allele compared to the -634 C-allele. Conversely, Watson et al.[149] have reported that the -634 C-allele results in lower VEGF production (~25% lower in heterozygotes; ~65% lower in CC homozygotes) in peripheral blood mononuclear cells (PBMCs) than the G-allele.

The G-1154A SNP is located in the promoter region of the *VEGF* gene, 1154bp upstream (5') of the canonical translation initiation codon[22]. Research has demonstrated that the A-allele at position -1154 results in ~25% lower *VEGF* gene expression in GI-1 glioma cells[82] and lower VEGF protein expression (~50% lower in AA homozygotes) in PBMCs[129] relative to the -1154 G-allele.

The C-2578A SNP is also located in the promoter region of the *VEGF* gene, 2578bp upstream (5') of the canonical translation initiation codon. The A-allele at position -2578 occurs with an 18-bp insertion at position -2549; when the C-allele is present at position -2578, no insertion is found[22]. Shahbazi et al. have shown that the -2578 A-allele results in significantly lower VEGF protein expression (~20% lower in heterozygotes; ~60% lower in AA homozygotes) in PBMCs when compared to the -2578 C-allele[129].

Investigations of VEGF gene haplotypes: While investigation of the effects of individual polymorphisms has been valuable, analysis of haplotypes (combinations of alleles at different, adjacent polymorphisms) is necessary to understand the overall function of the promoter region, as interaction among these individual polymorphisms likely plays a role in *VEGF* gene expression. There are 8 VEGF promoter region haplotypes incorporating the -2578, -1154, -634 SNPs, respectively: 4 common haplotypes (CGG, CGC, AGG, AAG; each with a frequency > 0.12) and 4 rare haplotypes (AAC, AGC, CAG, CAC; each with a frequency < 0.02).

One study of *VEGF* promoter region haplotypes used luciferase reporter vectors to assess V*EGF* haplotype influence on *VEGF* gene expression in the MCF7 (breast cancer) cell line[141]. Stevens et al.[141] investigated 3 different *VEGF* promoter region haplotypes, finding that the reporter with the AGG (-2578, -1154, and -634 alleles, respectively) haplotype resulted in higher *VEGF* gene expression than the AAG or CGC haplotypes. However, it is important to note these constructs differed from each other at one additional rare polymorphism that may have affected the results.

Another study has been performed in GI-1 glioma cells (human neural glial tumor cells)[82]. Two segments of *VEGF* DNA sequence from 5' position -2714 to -2469 and 5' position -1176 to -405 were ligated and inserted into a luciferase reporter vector. Subsequent luciferase assays revealed that the AAG and AGG haplotypes (-2578, -1154, and -634 alleles, respectively) resulted in lower *VEGF* gene expression relative to the CGC haplotype in both normoxia and hypoxia[82]. Additionally, Lambrechts et al.[82], assessed plasma VEGF levels among *VEGF* promoter region haplotypes in a sample of European patients with amyotrophic lateral sclerosis (ALS; also known as Lou Gehrig disease) and their spouses. The ALS patients of AAG/AAG, AGG/AGG, and AAG/AGG haplotypes exhibited lower plasma VEGF levels than all other haplotypes combined, but plasma VEGF levels were markedly lower in the group of ALS patients than in their spouses, and the haplotype associations observed in ALS patients were not statistically significant in the group of healthy spouses[82].

While the effects of these *VEGF* haplotypes have been demonstrated in specific cell types, they have not been investigated in skeletal muscle. It is currently unclear whether the aforementioned findings in glioma cells[82] or breast cancer cells[141] translate to tissue with greater relevance for Vo_{2max} such as skeletal muscle, where different transcription factors may regulate the *VEGF* gene. Likewise, it is not known whether the relationship between *VEGF* haplotypes and plasma VEGF levels would be observed in a more diverse sample of healthy individuals.

Furthermore, the effects of the *VEGF* promoter region haplotypes have not been investigated in the context of the continuous promoter region sequence without

the presence of additional genetic variation. In one experiment the AAG, AGG, and CGC haplotypes were studied, but the region from 5' position -2468 to -1177 (which contains the HRE) was absent in the reporter construct[82]. It is not known whether the same results would be observed if the region from 5' position -2468 to -1177 were present in the this experiment[82]. In another experiment, the same 3 haplotypes were studied in the continuous promoter region sequence, but additional genetic variation was present[141]. While the remaining haplotypes have been studied in relation to plasma VEGF levels in ALS patients, they have not been studied in healthy subjects and the effects of these haplotypes on *VEGF* gene expression are yet to be determined.

APPENDIX A – Limitations of the Study

Delimitations

Vo_{2max} and plasma VEGF protein levels:

1. Subjects recruited from the area surrounding the University of Maryland, College Park were evaluated for plasma VEGF protein levels and Vo_{2max}.

2. Subjects were black and white men and women, 50-75 years of age, sedentary, not diabetic, and free from cardiovascular disease. Therefore, the results are expected to apply to populations with similar characteristics.

3. Variables such as body composition[96], HRT[2,142], and sex[74] may have affects on VEGF protein expression, while body composition[100], HRT[113], sex[79,100], and age[54,57,100], may have affects on Vo_{2max} and ΔVo_{2max}. Therefore, statistical control for these variables was applied where appropriate.

4. Genotypes for polymorphisms other than the *VEGF* C-2578A, G-1154A, G-634C, C-7T, and C936T SNPs were not investigated for this study. Thus, it is possible that any haplotype effect on plasma VEGF levels, Vo_{2max}, or ΔVo_{2max} is due to linkage disequilibrium between these and other polymorphisms. Likewise, it is possible that the effects of these haplotypes are manifested only in the presence of an unknown combination of genetic factors.

5. Plasma VEGF levels were measured using a commercially available ELISA kit. Plasma samples were taken from fasting blood samples drawn during baseline testing.

6. Vo_{2max} was measured at baseline and after 24 weeks of aerobic exercise training using a customized metabolic system.

VEGF gene expression:

1. The effects of *VEGF* promoter region haplotype on *VEGF* gene expression were evaluated using cultured human myoblasts. Therefore, the results of this study are only expected to apply to human myoblasts grown in culture.

2. Recombinant plasmid vectors (pGL3-Basic luciferase reporter vectors with inserted *VEGF* promoter region) were transfected into cultured myoblasts using the Lipofectamine Plus Reagent (Invitrogen Corporation, Carlsbad, CA).

3. The Dual-Luciferase Reporter Assay System (Promega, Madison, WI) was used to quantify luciferase gene expression as a function of *VEGF* promoter region haplotype.

Limitations

Vo_{2max} and plasma VEGF protein levels:

1. Subjects self-reported many factors related to their health and lifestyle such as physical activity habits, medication regimens, and medical histories. It is possible that inaccurate self-reports have confounded the study results.

2. While each subject served as his own control for the analysis of ΔVo_{2max}, there was no non-exercising control group in this study. Thus it is possible that unknown effects of time have influenced the results of the analysis of ΔVo_{2max}.

3. The measurement of plasma VEGF levels was conducted on a single plasma sample. Therefore, it is possible that unknown daily variation in plasma VEGF level has influenced the study results.

4. Although skeletal muscle capillarity directly correlates with Vo_{2max}[50], the present study did not measure skeletal muscle capillarity before or after exercise training. Therefore, it is not possible to determine if the effects of the *VEGF* haplotypes on Vo_{2max} and ΔVo_{2max} are directly mediated through changes in skeletal muscle capillarity.

5. The present study did not measure plasma VEGF protein levels after acute or chronic aerobic exercise. Therefore, the effects of *VEGF* promoter region haplotypes on exercise-induced changes in VEGF protein expression were not assessed.

VEGF gene expression:

1. While VEGF is expressed in numerous human tissues, the present study has only assessed the effects of *VEGF* promoter region haplotypes in human myoblasts.

2. The human myoblasts used in this study were not obtained from the same subjects studied for Vo_{2max} and plasma VEGF levels, but from an independent sample of subjects. Due to sample availability, *VEGF* gene expression *in vitro* was only studied in myoblasts obtained from older white women.

3. *VEGF* gene expression as a function of *VEGF* promoter region haplotype was evaluated using a luciferase reporter vector transiently transfected into cultured human myoblasts. These cells were not stably transfected, nor was actual VEGF production among cells of different *VEGF* promoter region haplotypes determined.

APPENDIX B – Definition of Terms

3' untranslated region (3'UTR): the sequence of DNA that is transcribed and comprises the 3' end of mRNA, but is not translated into protein

5' untranslated region (5'UTR): the sequence of DNA that is transcribed and comprises the 5' end of mRNA, but is not translated into protein

Angiogenesis: the formation of blood vessels from pre-existing vessels

Capillary density: the number of capillaries in a given tissue cross section

Capillary to fiber ratio: the ratio of the number of capillaries to the number of skeletal muscle fibers in an area of tissue

Extracellular matrix (ECM): a complex array of proteins and polysaccharides that are secreted locally and form an organized meshwork in close association the cell surfaces that produced them

Haplotype: the arrangement of polymorphisms within a single chromosome: also considered to be the combination of alleles at polymorphisms in linkage disequilibrium with each other

Linkage Disequilibrium: the condition in which the haplotype frequencies in a population deviate from the values they would have if the alleles at each polymorphism were combined at random

Promoter Region: the region of DNA upstream (5') of the translation initiation codon where promoters and other transcription factors bind to regulate DNA transcription

Single nucleotide polymorphism (SNP): a DNA sequence variation involving the substitution of one nucleotide with a single, different nucleotide

Skeletal muscle capillarity: the number of capillaries present in an area of skeletal muscle; often expressed as capillary density or capillary to fiber ratio.

APPENDIX C – Human Subjects & Recombinant DNA Experiment Approval

This project has been approved by the Institutional Review Board at the University of Maryland, College Park as follows: 1) the project entitled: *VEGF gene sequence variation: Impacts on VEGF level and maximal oxygen consumption* (IRB# 05-0022, P.I. James M. Hagberg, Ph.D., Student Investigator Steven J. Prior, M.A.) and 2) the project entitled *VEGF gene sequence variation: Impacts on VEGF gene expression* (IRB# 05-0010, P.I. Stephen M. Roth, Ph.D., Student Investigator Steven J. Prior, M.A.). The corresponding IRB applications are reproduced in this Appendix.

Recombinant DNA experiments within the project entitled *VEGF gene sequence variation: Impacts on VEGF gene expression* have been approved by the Department of Environmental Safety at the University of Maryland, College Park (DES# 05-02).

VEGF gene sequence variation: Impacts on VEGF levels and maximal oxygen consumption

1. Abstract: The goal of this project will be to investigate the effects of variation in the DNA sequence of the vascular endothelial growth factor (*VEGF*) gene on maximal oxygen consumption (Vo_{2max}) and plasma VEGF protein levels. This project will involve: 1) genotyping current DNA holdings for *VEGF* polymorphisms, 2) biochemically analyzing levels of plasma VEGF protein in currently held plasma specimens, 3) obtaining previously existing Vo_{2max} data, and 4) conducting the appropriate statistical analysis. Each portion of the proposed project will utilize data and specimens previously collected from projects approved by the Institutional Review Board at the University of Maryland, College Park.

2. Subject Selection: The subjects for this project are those that have taken part in the ongoing and previously approved projects: *APO E Genotype and HDL Changes With Exercise Training* (IRB#00494, P.I. James M. Hagberg, Ph.D.) and *ACE Genotype, Blood Pressure, and Exercise Training in Hypertensives* (IRB#00736, P.I. James M. Hagberg, Ph.D.) from 1998 to the present time. No additional subjects will be recruited or tested for this project.

3. Procedures:

Genotyping: Standard genotyping methods will be used to genotype *VEGF* gene polymorphisms. All genomic DNA samples have been previously collected in

accordance with the IRB-approved projects above and stored in refrigeration in 2309 HHP Building.

Vo_{2max}: The investigators will obtain and analyze previously existing Vo_{2max} data, collected in the IRB-approved projects listed above.

Plasma VEGF Levels: The investigators will use previously existing plasma specimens banked as indicated in the projects noted above. Standard biochemical procedures will be used to determine VEGF protein levels in these specimens.

4. Risks and Benefits: There are no anticipated additional risks or benefits associated with the analysis of Vo_{2max} data beyond those which are outlined in the initial project applications. There are no additional risks or benefits associated with the determination of plasma VEGF levels as the samples have been previously obtained and no information regarding VEGF levels will be given to the subjects. There are no foreseen risks associated with genetic testing because the participants are not provided any information regarding their genetic testing results. This is necessary because the laboratories that perform these tests are not clinically certified and the information they provide can not be used for clinical or diagnostic purposes.

5. Confidentiality: No reference will be made to subject names in any presentations of the study results, including manuscripts. All data will be reported in the aggregate. The study data files are maintained in the office of the Study Coordinator for the aforementioned projects and access is provided only to qualified study personnel.

6. Information and Consent Forms: All subjects have provided written, informed consent as outlined in the project applications for the previously noted projects.

7. Conflict of Interest: Not applicable.

8. HIPAA Compliance: No information from the student health center is collected or used. No protected health information is collected or used beyond that which is collected for the original projects.

VEGF gene sequence variation: Impacts on *VEGF* gene expression

1. Abstract: The goal of this project will be to investigate the effects of variation in the DNA sequence of the vascular endothelial growth factor (*VEGF*) gene on *VEGF* gene expression in cultured tissue. This project will involve: 1) genotyping current DNA holdings for *VEGF* polymorphisms, 2) culturing previously obtained human skeletal muscle samples, 3) carrying out DNA transfection in the cultured skeletal muscle samples, 4) assessing expression of a reporter vector in the transfected cells, and 5) conducting the appropriate statistical analysis. Each portion of the proposed project will utilize specimens previously collected from projects approved by the Institutional Review Board at the University of Maryland, College Park (Studies of Human Genetic Variation, IRB#01198, P.I.: Stephen M. Roth, Ph.D.), or by the Institutional Review Board at the University of Maryland School of Medicine (Race, Menopause, and Metabolism After Exercise and Diet, IRB#M1174, P.I.: Alice S. Ryan, Ph.D.).

2. Subject Selection: The DNA samples to be used in this project were originally obtained as part of the project, *Studies of Human Genetic Variation*. Samples will be selected for use based on the specific combination of alleles in and surrounding the *VEGF* gene. This sample selection will require no further contact with, or information from, human subjects.

The human muscle samples to be used in this project have been collected as described in the project, *Race, Menopause, and Metabolism After Exercise and Diet*.

Beyond that, samples will be selected for use based on the success of their growth and transfection *in vitro*. No new subjects will be recruited or tested; the proposed project involves only an analysis of existing biological specimens.

3. Procedures: The collection of DNA samples was conducted as described in the project, *Studies of Human Genetic Variation*. For the current project, samples will be genotyped for several polymorphisms in the *VEGF* gene. Small fragments (less than 4000 nucleotides) of isolated DNA will be amplified from selected samples using standard polymerase chain reaction techniques. This fragment will then be inserted into a reporter vector and passed into cells cultured *in vitro* using standard recombinant DNA techniques. Cells cultured in vitro will be of human nature and have been obtained as part of the project, *Race, Menopause, and Metabolism After Exercise and Diet.* These cells will be immediately destroyed following the experiment.

4. Risks and Benefits: There are no anticipated additional risks or benefits associated with this project beyond those which are outlined in the initial project applications. All samples have been previously obtained and no information regarding study results will be given to the subjects. There are no foreseen risks associated with genetic testing because the participants are not provided any information regarding their genetic testing results. This is necessary because the laboratories that perform these tests are not clinically certified and the information they provide can not be used for clinical or diagnostic purposes.

5. Confidentiality: For samples collected in the project, *Studies of Human Genetic Variation*, no identifying information has been obtained from any volunteer, ensuring confidentiality. The data files for the project, *Race, Menopause, and Metabolism After Exercise and Diet* are maintained in the study office and access is provided only to qualified study personnel. No reference will be made to subject names in any presentations of the study results, including manuscripts.

6. Information and Consent Forms: All subjects have provided written, informed consent as outlined in the project applications for the previously noted projects.

7. Conflict of Interest: Not applicable.

8. HIPAA Compliance: No information from the student health center is collected or used. No protected health information is collected or used beyond that which is collected for the original projects.

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