DNA sequence variation in the promoter region of the VEGF gene impacts VEGF gene expression and maximal oxygen consumption

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Prior, Steven J., James M. Hagberg, Chad M. Paton, Larry W. Douglass, Michael D. Brown, John C. McLenithan, and Stephen M. Roth. DNA sequence variation in the promoter region of the VEGF gene impacts VEGF gene expression and maximal oxygen consumption. Am J Physiol Heart Circ Physiol 290: H1848–H1855, 2006. First published December 9, 2005; doi:10.1152/ajpheart.01033.2005.—In its role as an endothelial cell proliferation and migration factor, vascular endothelial growth factor (VEGF) can affect peripheral circulation and therefore impact maximal oxygen consumption (V\textsubscript{O2 max}). Because of the role of VEGF, and because variation in the VEGF gene has the ability to alter VEGF gene expression and VEGF protein level, we hypothesized that VEGF gene polymorphisms are related to VEGF gene expression in human myoblasts and V\textsubscript{O2 max} before and after aerobic exercise training. We analyzed the effects of the VEGF −2578/−1154/−634 promoter region haplotype on VEGF gene expression by using a luciferase reporter assay in cultured human myoblasts and found that the AAG and CGC haplotypes resulted in significantly higher hypoxia-stimulated VEGF gene expression than the AAG and CGC haplotypes. Consistent with these results, we found that individuals with at least one copy of the AAG or CGC haplotype had higher V\textsubscript{O2 max} before and after aerobic exercise training than did subjects with only the AGG and/or CGG haplotype. In conclusion, we found that VEGF −2578/−1154/−634 haplotypes impact VEGF gene expression in human myoblasts and is associated with V\textsubscript{O2 max}. These results have potential implications for aerobic exercise training and may prove relevant in the study of pathological conditions that can be affected by angiogenesis, such as coronary artery disease and peripheral artery disease.

angiogenesis; exercise; genetics; polymorphism

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 (CAD) (33) and peripheral artery disease (PAD) (11)] and physiological conditions [e.g., aerobic exercise (5)] as blood flow to tissues is often limited. This is especially relevant in human skeletal muscle where blood flow, and thus oxygen supply, can limit maximal oxygen consumption (V\textsubscript{O2 max}) (36). V\textsubscript{O2 max} is inversely associated with cardiovascular and all-cause mortality (8), a relationship that has been well documented in a variety of populations. It is also well established that V\textsubscript{O2 max} is responsive to aerobic exercise training, such that V\textsubscript{O2 max} typically increases ~15–30% after 3–9 mo of training (23, 40). Concordantly, improvement in cardiorespiratory fitness has been shown to result in decreased risk of cardiovascular disease mortality and all-cause mortality (8). As the genesis of new vasculature can influence V\textsubscript{O2 max} by increasing local circulation and oxygen supply, investigation of the mechanisms underlying the process of angiogenesis is of significant clinical interest.

Angiogenesis is a critical phenomenon in the adaptation to aerobic exercise training because a contributing mechanism to the training-induced increase in V\textsubscript{O2 max} is an increase in skeletal muscle capillarity (5). This is important because a proportion of the increase in V\textsubscript{O2 max} with training is attributed to increased oxygen extraction by the working muscle (35, 40). Angiogenesis can contribute to this increase in oxygen extraction by increasing the capillary surface area for diffusion, decreasing the average oxygen diffusion path length in skeletal muscle and increasing red blood cell transit time through skeletal muscle (31). Increases in arteriovenous oxygen difference have been observed after aerobic exercise training and are responsible, in part, for training-induced increases in V\textsubscript{O2 max} in older individuals (13, 45, 51).

Aerobic exercise training has been identified as a powerful angiogenic stimulus as studies over the last three decades have shown increases in skeletal muscle capillarity up to ~30% in as little as 1–3 mo of training (1, 44). Vascular endothelial growth factor (VEGF) has been identified as one of the key regulators of angiogenesis because it plays a role in endothelial cell proliferation (14) and migration (18). VEGF is expressed in numerous human tissues, including skeletal muscle, and recent research indicates that VEGF is indeed involved in the angiogenic response to aerobic exercise (15, 38). For example, aerobic exercise creates a hypoxic condition in skeletal muscle [P\textsubscript{O2} ~2–4 Torr (37, 49)] that upregulates VEGF gene transcription and increases the half-life of VEGF mRNA through hypoxia inducible factor-1 (27).

Significant variability has been observed among similar individuals in V\textsubscript{O2 max} (4), skeletal muscle capillarity (5, 44), and VEGF gene expression (42). For example, Schultz et al. (42) have demonstrated a range of ~1- to 7-fold hypoxic induction of VEGF mRNA expression in monocytes derived from CAD patients. 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 (42).

Although some proportion of the variability in the aforementioned traits can doubtlessly be attributed to nongenetic factors, there appears to be a significant contribution of genetic factors.

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Twin studies have revealed significant correlations of $V_{O2\text{ max}}$ between sibling pairs (3, 22), and additional research has provided heritability estimates for $V_{O2\text{ max}}$ and the response of $V_{O2\text{ max}}$ to aerobic exercise training as high as 59% and 47%, respectively, although it is recognized that nongenetic familial influences also contribute to these heritability estimates (2). Although the genetic contribution to skeletal muscle capillarity has yet to be defined, investigators have argued that differences in the vasculature among individuals can be attributed to both environmental factors (e.g., aerobic exercise training) and genetic factors (5, 34). 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 (25, 47), indicating a genetic contribution to VEGF gene expression.

DNA sequence variation in the promoter region of VEGF gene has previously been associated with differences in VEGF gene and protein expression. The C-2578A (43) [which is linked to an 18-bp insertion/deletion polymorphism at position \(-2549\) (6)], G-1154A (43), and G-634C (50) single nucleotide polymorphisms (SNPs) in the promoter region of the VEGF gene have been associated with VEGF protein expression in peripheral blood mononuclear cells, and the VEGF \(-2578/-1154/-634\) haplotype has been associated with VEGF gene expression in cultured GI-1 glioma cells (25), as well as in MCF7 breast cancer cells (47).

**METHODS**

**Purposes**

As the effects of the VEGF promoter region haplotypes have not been investigated in skeletal muscle (a tissue highly relevant for $V_{O2\text{ max}}$), where different factors may regulate VEGF gene expression, the purpose of this project was to investigate the VEGF \(-2578/-1154/-634\) promoter region haplotype for effects on VEGF gene expression in cultured skeletal muscle cells and for association with $V_{O2\text{ max}}$ before and after aerobic exercise training.

In addition, the utility of measuring plasma VEGF levels is currently unknown. Plasma VEGF levels may be indicative of angiogenic activity (21) and may be influenced by acute exercise (16, 24), but the relevance of circulating VEGF protein is not clear. An association has been reported between VEGF \(-2578/-1154/-634\) haplotype and plasma VEGF levels in subjects with amyotrophic lateral sclerosis (ALS) (25), but this remains to be observed in healthy subjects. As it is currently unknown whether plasma VEGF levels are reflective of skeletal muscle VEGF expression or are predictive of $V_{O2\text{ max}}$, we investigated whether an association exists between plasma VEGF level and VEGF \(-2578/-1154/-634\) haplotype, and whether a correlation exists between plasma VEGF level and $V_{O2\text{ max}}$.

**METODOLOGY**

**Subjects.** $V_{O2\text{ max}}$ and VEGF haplotype data were available for 148 white and black human subjects. Baseline plasma VEGF level was measured in 92 subjects. Some subjects used for the study of plasma VEGF level did not complete exercise training and were not used in the study of $V_{O2\text{ max}}$ (as indicated by different numbers of subjects in Table 1). Selection of subjects for the study of $V_{O2\text{ max}}$ and plasma VEGF levels has been previously described (52). Briefly, all subjects were required to 1) be sedentary (exercise <20 min, 2 times/wk, for at least 6 mo); 2) be 50–75 yr of age; 3) not be taking lipid- or glucose-lowering medication; 4) be normotensive or hypertensive (systolic blood pressure between 121–160 mmHg and/or diastolic blood pressure 81–99 mmHg) 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 (BMI) <37 kg/m²; and 9) not have any other medical condition that would preclude vigorous aerobic exercise. Additionally, all female participants were at least 2 yr past menopause and agreed to maintain their hormone replacement therapy status for the duration of the study. Skeletal muscle biopsies for the VEGF gene expression experiments were obtained from three healthy white women (age 58 ± 4.4 yr; BMI 28.9 ± 3.4 kg/m²). Selection of these subjects was similar and has also been previously described (41). DNA for the VEGF gene expression experiments was obtained from subjects recruited to donate DNA samples for in vitro experimentation. All subjects gave written informed consent and all study protocols were approved by the Institutional Review Boards at the University of Maryland and University of Maryland School of Medicine.

**Exercise training.** The aerobic exercise training protocol has been previously described (52). Briefly, subjects underwent 24 wk of standardized aerobic exercise training. Subjects began at a training volume of three sessions of 20 min at 50% of heart rate reserve (HRreserve) per week and gradually increased to three sessions of 40 min at 70% of HRreserve per week, a level maintained for the final 14 wk of the intervention. For inclusion in the analyses of $V_{O2\text{ max}}$ after training, subjects were required to have completed at least 75% of the scheduled exercise sessions.

**$V_{O2\text{ max}}$ and body composition.** $V_{O2\text{ max}}$ was assessed as previously described (52). Briefly, $V_{O2\text{ max}}$ was measured continuously with a validated system during a graded exercise test on a treadmill to maximal effort. The test consisted of a continuous series of 2-min stages, where speed was fixed and treadmill grade was increased 2% in each stage. Percent body fat was measured at baseline using dual-energy X-ray absorptiometry (DPX-L; Lunar, Madison, WI).

**Plasma VEGF.** During baseline testing, fasting blood samples were collected for the 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. Each plasma sample was measured in duplicate, and the

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**Table 1. Characteristics of subjects**

<table>
<thead>
<tr>
<th></th>
<th>Study of $V_{O2\text{ max}}$</th>
<th>Study of Plasma VEGF Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>Men ($n = 61$)</td>
<td>Women ($n = 85$)</td>
</tr>
<tr>
<td>Height, cm</td>
<td>59.1 ± 0.8</td>
<td>57.7 ± 0.5</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>28.5 ± 0.5</td>
<td>28.2 ± 0.5</td>
</tr>
<tr>
<td>Baseline weight, kg</td>
<td>90.1 ± 1.8</td>
<td>75.6 ± 1.4</td>
</tr>
<tr>
<td>Change in Weight, kg</td>
<td>-1.8 ± 0.3</td>
<td>-1.1 ± 0.2</td>
</tr>
<tr>
<td>Baseline $V_{O2\text{ max}}$ ml/kg•min⁻¹</td>
<td>28.2 ± 0.6</td>
<td>22.4 ± 0.4</td>
</tr>
<tr>
<td>Plasma VEGF level, pg/ml</td>
<td>17.1 ± 1.1 ($n = 27$)</td>
<td>17.9 ± 1.5 ($n = 33$)</td>
</tr>
<tr>
<td>Data are means ± SE. $V_{O2\text{ max}},$ maximal O$_2$ consumption; BMI, body mass index; VEGF, vascular endothelial growth factor.</td>
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average of those two measurements was used for statistical analyses.
Soluble VEGF receptor-1 (sVEGFR-1) levels were measured by using the sVEGFR R1 (Flt-1) Quantikine ELISA Kit (R&D Systems) to determine if interference with VEGF level measurement existed (20). No sVEGFR-1 interference was detected in any sample.

**VEGF genotype and haplotype determination.** Genomic DNA was extracted from peripheral lymphocytes of whole blood samples by using the PureGene DNA extraction kit (Gentra Systems). The VEGF G-634C SNP was genotyped by restriction fragment length polymorphism (RFLP) analysis by using the forward PCR primer 5‘-GTA GCA AGA GCT CCA GAG AGT-3‘, the reverse PCR primer 5‘-TGG ACG AAA AGT TTC AGT GCG ACG-3‘, and the BsmFI restriction endonuclease. The VEGF G-1154A SNP was genotyped by pyrosequencing (39) by using the forward PCR primer 5‘-GTC GAG CTT CCC CTT CAT T-3‘, the reverse PCR primer 5‘-CCG CTA CCA GCC GAC TTT T-3‘, and the internal pyrosequencing primer 5‘-AGC CGC GTG TGG A-3‘. Genotypes were detected by using the Pyrosequencing PSQ HS 96 light detection system (Pyrosequencing AB, Uppsala, Sweden). The VEGF C-2578A SNP was genotyped by RFLP analysis by using the forward PCR primer 5‘-CTG ACT AGG TAA GCT CCC TGG A-3‘, the reverse PCR primer 5‘-AGC CCC CTT TCC CCA CT-3‘, and the BglII restriction endonuclease.

**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 by 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 separate 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 BglII to determine the −2578 allele that is on the same chromosome as either the −634 C-allele or G-allele. This method of determining the cis/trans configuration of VEGF haplotypes was validated, and haplotypes were confirmed by sequencing the allelespecific PCR amplimers from step 1 in a sample of 96 subjects.

**Cell culture and transfection.** Pericutaneous biopsies of the vastus lateralis muscle were obtained from the subjects, and primary cultures of human myoblasts were generated by using a method similar to that described by Thompson et al. (48). Primary myoblast cultures were subcultured and plated onto 24-well BioCoat Collagen I-coated culture plates (BD Biosciences Discovery Labware, Bedford, MA) and grown in Skeletal Muscle Basal Medium (Cambrex, East Rutherford, NJ) supplemented with 10% fetal bovine serum and incubated at 37°C with 95% air-5% CO2. The cell culture medium was changed every other day to achieve 50–70% confluence on the day of transfection. Thawed cell samples were split into no more than a 1:1.5 ratio, and cells used for this experiment were no greater than passage 3. For each experimental construct (i.e., for each different VEGF haplotype), six samples were transfected: two samples from each of the three different primary cultures from different individuals. Transfection of myoblasts was conducted with the Lipofectamine Plus Reagent (Invitrogen) by using 2 µl of Lipofectamine, 4 µl of Plus Reagent, 0.4 µg of recombinant pGL3-Basic vector containing one VEGF promoter region haplotype, and 0.02 µg of pRL-CMV vector per well (to normalize for transfection efficiency). After 3 h of incubation, the media with transfection agents were removed and replaced with the aforementioned media.

Two incubation conditions were used after transfection: normoxia (20% ambient O2) and hypoxia (1% ambient O2). All cells were initially incubated as noted above for 32 h. For the normoxic condition, transfected cells were maintained in these conditions (20% O2) for an additional 16 h. For the hypoxic condition, cell culture plates were placed in a 5310 Desiccator (Nalgene Labware, Rochester, NY). The desiccator was flushed with a low-oxygen gas mix (1% O2-5% CO2-94% N2) for 20 min and then sealed and incubated at 37°C in a method similar to that described by Forsythe et al. (10). After 1 h, the desiccator was again flushed with low-oxygen gas for 10 min to account for residual air that may have remained in the culture plates after the first flush. The desiccator was then sealed and incubated at 37°C for 15 h.

**Luciferase activity.** The Dual-Luciferase Reporter Assay System (Promega, Madison, WI) was used to quantitate luciferase signals from transfected myoblasts. Firefly luciferase activity was determined by mixing 20 µl of cell lysate with 100 µl of Luciferase Assay Reagent II and measurement of relative light units with a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA). Renilla luciferase activity was measured by the addition of 100 µl of Stop & Glo Reagent and subsequent measurement of relative light units. Each 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.** The gene expression data were analyzed using a two-factor (2 × 4: %O2 × haplotype) factorial ANOVA.
VEGF HAPLOTYPE IMPACTS VEGF GENE EXPRESSION AND VO2\text{max}

Table 2. VEGF polymorphism statistics

<table>
<thead>
<tr>
<th>VEGF Polymorphism Allele Frequencies</th>
<th>White Subjects (n = 119)</th>
<th>Black Subjects (n = 29)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p</td>
<td>q</td>
</tr>
<tr>
<td>C-2578A</td>
<td>0.51 (A)</td>
<td>0.49 (C)</td>
</tr>
<tr>
<td>G-1154A</td>
<td>0.67 (G)</td>
<td>0.33 (A)</td>
</tr>
<tr>
<td>G-634C</td>
<td>0.73 (G)</td>
<td>0.27 (C)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>VEGF -2578/−1154/−634 Haplotype Frequencies</th>
<th>CGG</th>
<th>CGC</th>
<th>AAG</th>
<th>AGG</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>White subjects (n = 119)</td>
<td>0.21</td>
<td>0.27</td>
<td>0.32</td>
<td>0.19</td>
<td>0.01</td>
</tr>
<tr>
<td>Black subjects (n = 29)</td>
<td>0.67</td>
<td>0.19</td>
<td>0.05</td>
<td>0.19</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Linkage Disequilibrium Values (r²) for VEGF Polymorphisms

<table>
<thead>
<tr>
<th></th>
<th>−2578</th>
<th>−1154</th>
</tr>
</thead>
<tbody>
<tr>
<td>−1154</td>
<td>0.39*</td>
<td></td>
</tr>
<tr>
<td>−634</td>
<td>0.23*</td>
<td>0.10*</td>
</tr>
</tbody>
</table>

*P < 0.01.

RESULTS

The VEGF −2578/−1154/−634 haplotype was studied for effects on VEGF gene expression and for association with VO2\text{max} before and after aerobic exercise training. All haplotype groups exhibited significant increases in VO2\text{max} before (P < 0.01) after aerobic exercise training. In general, subjects experienced modest but statistically significant weight loss (~1.4 kg, P < 0.05), but no significant differences in weight loss were observed among haplotype groups. Characteristics of subjects used for the study of VO2\text{max} and for the study of plasma VEGF level are shown in Table 1.

Allele and haplotype frequencies. Allele and haplotype frequencies were calculated from 148 individuals (Table 2). Four common haplotypes (CGG, CGC, AAG, and AGG) comprised ~99% of all observed haplotypes and were subsequently studied. Those subjects with one of the other four possible haplotypes (AGC, AAC, CAC, or CAG; n = 2) were not included in the analyses of VO2\text{max} or plasma VEGF. With the exception of the G-634C polymorphism (P = 0.02), no significant deviation from Hardy Weinberg equilibrium was observed (P = 0.38–0.40). Differences in allele frequencies between race groups were observed, with black subjects typically having lower frequency of the rare allele. Moderate but statistically significant linkage disequilibrium was detected among the three SNPs (Table 2).

VEGF gene expression. We conducted an experiment with the four common VEGF −2578/−1154/−634 haplotypes to assess potential effects on VEGF gene expression in human myoblasts. Under normoxic (~20% O2) conditions, the AAG haplotype resulted in significantly lower luciferase activity than the CGG haplotype (P = 0.02), and there were tendencies for the AGG and CGC haplotypes to result in lower luciferase activity than the CCG haplotype (Fig. 2; P = 0.065 and P = 0.079, respectively). Under hypoxic (~1% O2) conditions, the CCG haplotype resulted in significantly lower luciferase activity than the AAG and CGC haplotypes (Fig. 2; P = 0.006 and P = 0.002, respectively). The hypoxia-stimulated increase in luciferase activity was markedly different among the four haplotypes, with the CGG and AGG haplotypes resulting in lower hypoxic induction (1.5-fold and 2.0-fold increases, respectively) than the AAG and CGC haplotypes (3.1-fold and 3.2-fold increases, respectively).

VO2\text{max}. On the basis of the results of the VEGF gene expression experiments under hypoxic conditions, subjects were grouped by VEGF −2578/−1154/−634 haplotype (Table 3) to test for associations with VO2\text{max}. We hypothesized that group 1 (AGG/AGG, AGG/CGG, and CGG/CGG haplotype combinations) would exhibit lower VO2\text{max} before and after exercise training compared with group 2 (AGG/AAG, AGG/CGC, CGG/AAG, and CGG/CGC haplotype combinations) and group 3 (AAG/AAG, AAG/CGC, and CGC/CGC haplotype combinations) and that group 2 would exhibit lower VO2\text{max} before and after exercise training compared with group 3. The results supported the hypothesis that group 1 had lower VO2\text{max} before (P = 0.021 and P = 0.038) and after (P = 0.006 and P = 0.010) exercise training than groups 2 and 3, respectively, and group 1 tended to have a lower ΔVO2\text{max} with training compared with groups 2 and 3 (P = 0.063 and P = 0.062, respectively). No significant differences were observed between groups 2 and 3. All results are shown in Table 3.
The principal findings of the present study are that 1) VEGF−2578/−1154/−634 haplotype impacted VEGF gene expression in human myoblasts under hypoxic conditions in vitro; 2) the VEGF−2578/−1154/−634 haplotypes impacting VEGF gene expression had a concordant association with VO₂ max in older individuals before and after a standardized program of aerobic exercise training; and 3) plasma VEGF level in older individuals was not associated with VEGF−2578/−1154/−634 haplotype, nor did plasma VEGF level correlate with baseline VO₂ max or ΔVO₂ max with aerobic exercise training. To our knowledge this is the first report that VEGF−2578/−1154/−634 haplotype impacts VEGF gene expression in cultured human myoblasts and is associated with VO₂ max.

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. In normoxic (~20% O₂) conditions, the CGG haplotype appeared to result in ~40% higher VEGF gene expression in cultured human myoblasts than the CGC, AGG, and AAG haplotypes. However, the condition of ~20% O₂ in the tissue culture incubator is considered by some to be supranormal and has been shown to elevate the PO₂ in cultured mouse skeletal muscle cells to ~40 Torr (approximately twice the normal level in mice and other mammals) (9). Such an elevation of PO₂ in our cultured human myoblasts may have impacted the reporter expression we observed, so it is not known whether the normoxic 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.

To better simulate PO₂ in exercising skeletal muscle, we incubated myoblasts under conditions of hypoxia (1% O₂). Eu et al. (9) have found that incubation in 1% O₂ results in a PO₂ of ~3.5 Torr in mouse skeletal muscle cells, a level that is representative of PO₂ in exercising skeletal muscle [2–4 Torr (36) and that is low enough to induce hypoxic activation of VEGF gene transcription (10, 27)]. Our experiment showed that under hypoxic conditions, the AAG and CGC haplotypes resulted in ~43% higher VEGF gene expression than did the CGG and AGG haplotypes.

In this hypoxic experiment, the CGG and AGG haplotypes exhibited the lowest hypoxic induction (1.5-fold and 2.0-fold, respectively), and the AAG and CGC haplotypes exhibited the highest hypoxic induction of VEGF gene expression (3.1-fold and 3.2-fold, respectively). Generally, this induction was within the range of increases in VEGF mRNA (2- to 6-fold) observed by others (12, 15, 38) after acute exercise in vivo.

### Table 3. VO₂ max values for subjects grouped by VEGF−2578/−1154/−634 haplotype

<table>
<thead>
<tr>
<th>VEGF−2578/−1154/−634 Haplotype</th>
<th align="right">1 (n = 37)</th>
<th align="right">2 (n = 65)</th>
<th align="right">3 (n = 44)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline VO₂ max, ml·kg⁻¹·min⁻¹</td>
<td align="right">23.9 ± 0.58</td>
<td align="right">25.4 ± 0.53</td>
<td align="right">25.3 ± 0.62</td>
</tr>
<tr>
<td>Final VO₂ max, ml·kg⁻¹·min⁻¹</td>
<td align="right">26.9 ± 0.74</td>
<td align="right">29.4 ± 0.68</td>
<td align="right">29.4 ± 0.80</td>
</tr>
<tr>
<td>ΔVO₂ max, ml·kg⁻¹·min⁻¹</td>
<td align="right">3.02 ± 0.47</td>
<td align="right">3.93 ± 0.36</td>
<td align="right">3.80 ± 0.43</td>
</tr>
</tbody>
</table>

Haplotypes included: AGG/AGG, AGG/CGG, CGG/AGG, AGG/CGG, CGG/CGG, CGG/CGC, CGG/CGA.

Data are means ± SE. a,b,c,d Significant difference between values with like superscript. *Trend for ΔVO₂ max, to be lower in group 1 compared with groups 2 and 3.

### Table 4. Plasma VEGF levels for subjects grouped by VEGF−2578/−1154/−634 promoter region haplotype indicated by gene expression results in normoxia and gene expression results in hypoxia

<table>
<thead>
<tr>
<th>Gene Expression in Normoxia: VEGF−2578/−1154/−634 Haplotype</th>
<th align="right">A (n = 22)</th>
<th align="right">B (n = 70)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma VEGF level, pg/ml</td>
<td align="right">18.5 ± 2.1</td>
<td align="right">17.8 ± 1.3</td>
</tr>
</tbody>
</table>

Haplotypes included: CGG/CGG, All others

<table>
<thead>
<tr>
<th>Gene Expression in Hypoxia: VEGF−2578/−1154/−634 Haplotype</th>
<th align="right">1 (n = 30)</th>
<th align="right">2 (n = 47)</th>
<th align="right">3 (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma VEGF level, pg/ml</td>
<td align="right">17.2 ± 1.7</td>
<td align="right">19.3 ± 1.5</td>
<td align="right">16.0 ± 2.5</td>
</tr>
</tbody>
</table>

Haplotypes included: AGG/AGG, AGG/AGG, CGG/AGG, AGG/CGC, CGG/CGG, CGG/CGG, CGG/CGA.

Data are adjusted LS means ± SE. All means are adjusted for age, race, and percent body fat. For normoxia, P = 0.40 for haplotype group effect. For hypoxia, P = 0.20 for haplotype group effect.
was expected that the hypoxia-induced VEGF gene expression in our experiment would be at the lower end of the range of VEGF mRNA increase in vivo for two reasons. First, the 3'-untranslated region and coding sequence of the VEGF gene was not incorporated into the experimental reporter vector. Thus, although the hypoxia induction of VEGF gene transcription observed in vivo appears to have occurred in these reporter vectors, the hypoxia-mediated stabilization of VEGF mRNA (19, 26, 46) would not be expected in these reporter vectors in vitro, likely resulting in less accumulation of reporter protein. 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 (28), tumor necrosis factor-α (30), and AMP-activated protein kinase (29) likely contribute to the larger VEGF mRNA increase observed in vivo.

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., AGG and CGG haplotypes) appears to result in lower VEGF gene expression in cultured human myoblasts relative to the other two haplotypes (AAG and CGC); the presence of the A- or C-allele at the −2578 SNP (i.e., the first position in the haplotype) did not appear to affect the observed VEGF gene expression. Our data indicate that the effect of these haplotypes would fit a dominant/recessive genetic model given that only subjects with two copies of the AGG or CGG haplotypes exhibit low \( V_{O_2\max} \) whereas subjects with \( \geq 1 \) copy of the AAG or CGC haplotypes exhibit higher \( V_{O_2\max} \).

As none of these three polymorphisms occur within any specific transcription factor binding site identified to date (30), the precise mechanism for VEGF −2578/−1154/−634 haplotype effects on VEGF gene expression is still not known. The possibility exists that these polymorphisms disrupt transcription factor binding sites that have not yet been defined or affect interactions among transcription factors. For example, the hypoxia response element (HRE) in the VEGF promoter region (5'-position −2012 to −2005) requires interaction with an upstream activator protein-1 (5'-position −2166 to −2160) and downstream activator protein-2α (5'-position −1117 to −1110) (30) that lie within the promoter region sequence we tested; we speculate that the −1154 and/or −634 SNPs may affect these interactions in some manner.

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 −2578/−1154/−634 haplotypes have been performed in other cell lines. Stevens et al. (47) investigated three VEGF −2578/−1154/−634 haplotypes (AGG, AAG, and CGC) in MCF7 breast cancer cells by 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 (from that used in the present study) at additional polymorphisms, which makes comparing the present and former reports difficult. Lambrechts et al. (25) studied the same three VEGF −2578/−1154/−634 haplotypes in GI-1 glioma cells, demonstrating that the AAG and AGG haplotypes resulted in lower VEGF gene expression relative to the CGC haplotype in both normoxic and hypoxic conditions; however, the region from 5'-position −2468 to −1177 (which contains the HRE) was absent in their reporter construct. Although 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).

\( V_{O_2\max} \). The results of this investigation also demonstrate an association between VEGF −2578/−1154/−634 haplotype and \( V_{O_2\max} \) in older individuals. We recognize that the genotype frequencies for the −634 SNP in our subjects differed from HWE expectations, but our genotype and haplotype frequencies are similar to those from previous reports (25), so we do not believe that this significantly impacted our results.

On the basis of the gene expression studies of these haplotypes under hypoxic conditions, subjects were grouped for analysis of association between VEGF −2578/−1154/−634 haplotype and \( V_{O_2\max} \). We chose to group subjects on the basis of the hypoxic gene expression results for two reasons. First, the normoxic condition in the tissue culture incubator is not likely to reflect the Po2 in resting skeletal muscle. Second, we speculate that differences in \( V_{O_2\max} \) 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 \( V_{O_2\max} \) indicate that subjects with only CGG and/or AGG haplotypes (group 1) exhibited significantly lower \( V_{O_2\max} \) 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 \( \Delta V_{O_2\max} \). These relationships are in agreement with the results of the VEGF gene expression experiments, suggesting a functional effect of these polymorphisms in older individuals. The most likely mechanism to explain the relationship between VEGF −2578/−1154/−634 haplotype and \( V_{O_2\max} \) is that VEGF −2578/−1154/−634 haplotype impacts VEGF gene expression, resulting in 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. In the present study, we have observed an association between VEGF −2578/−1154/−634 haplotype and \( V_{O_2\max} \) and have begun to address the mechanism of this association by demonstrating a concordant influence of these haplotypes on VEGF gene expression in human myoblasts. Because neither skeletal muscle VEGF protein expression in vivo nor skeletal muscle capillarity data were available for the subjects in this report, these remain to be addressed in future research.

**Plasma VEGF level.** Analyses of plasma VEGF level as a function of VEGF −2578/−1154/−634 haplotype and in relation to \( V_{O_2\max} \) 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 \( V_{O_2\max} \) and \( \Delta V_{O_2\max} \) 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 \( V_{O_2\max} \). Our results are in partial disagreement with
those of Lambrechts et al. (25). Their group found that VEGF 
−2578/−1154/−634 haplotype was associated with plasma 
VEGF levels in a sample of European patients with ALS such 
that patients with AAG and/or AGG haplotypes exhibited 
lower plasma VEGF levels than all other haplotypes combined, 
but this association was not significant in the healthy spouses 
of these patients (25).

Because there is still some question as to the relevance 
of plasma VEGF level, we do not feel that the lack of association 
between VEGF haplotype and plasma VEGF level detracts 
from the other findings in this report. As the relative contribu-
tion of specific cell types to plasma VEGF levels is not known, 
it is possible that skeletal muscle is a minor contributor to 
plasma VEGF under resting conditions. This could explain 
the lack of association between VEGF haplotype and plasma 
VEGF level at rest. Additionally, because plasma VEGF 
levels did not correlate with $\dot{V}_{O_2}$ max, our results indicate 
that plasma VEGF level under resting conditions may not be 
a relevant intermediate phenotype between VEGF haplotype 
and $V_{O_2}$ max.

Hiscock et al. (16) and Kraus et al. (24) have reported 
increases in plasma VEGF after an acute bout of exercise, 
indicating that acute exercise influences plasma VEGF level, 
but the relevance of that protein is still unresolved. Kraus et al. 
(24) have shown that plasma VEGF level increases at 0 and 2 h 
after aerobic exercise with no difference from baseline at 4 h. 
It has also been shown that skeletal muscle VEGF protein 
content is decreased at 0 h after exercise and returns to 
near-baseline levels at 4 h (12). This temporal relationship, 
coupled with the observation that VEGF protein increases 
in the skeletal muscle interstitium during acute exercise (17), 
suggests that skeletal muscle VEGF expression and secretion 
could impact plasma VEGF after acute exercise. Whether 
plasma VEGF level after acute exercise is reflective of VEGF 
gene expression in skeletal muscle or predictive of $V_{O_2}$ max 
remains to be seen. The measures of plasma and skeletal 
muscle VEGF protein after acute exercise needed to address 
these questions were not available for our subjects, but these 
measures should be addressed in the future to clarify the 
relationships between skeletal muscle, plasma VEGF, and 
angiogenesis.

In summary, the current study shows an impact of the VEGF 
−2578/−1154/−634 haplotype on VEGF gene expression in 
cultured human myoblasts and on $V_{O_2}$ max before and after 
exercise training such that the AGG and CGG haplotypes 
were related to lower hypoxia-stimulated VEGF gene expression 
and lower $V_{O_2}$ max in older individuals compared with the AAG 
and CGC haplotypes. Although factors other than hypoxia and 
VEGF certainly contribute to $V_{O_2}$ max, before and after aerobic 
exercise training, these VEGF haplotype groups were associated 
with −8–10% differences in $V_{O_2}$ max both before and after 
24 wk of aerobic exercise training. These results have potential 
implications for aerobic exercise training and the risk of 
morbidity/mortality that is associated with cardiorespiratory 
fitness (i.e., $V_{O_2}$ max). Additionally, our findings may 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, CAD, and PAD.

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