Vascular endothelial growth factor mRNA expression and arteriovenous balance in response to prolonged, submaximal exercise in humans

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Hiscock, N., C. P. Fischer, H. Pilegaard, and B. K. Pedersen. Vascular endothelial growth factor mRNA expression and arteriovenous balance in response to prolonged, submaximal exercise in humans. Am J Physiol Heart Circ Physiol 285: H1759–H1763, 2003. First published May 22, 2003; 10.1152/ajpheart.00150.2003.—Angiogenesis, the formation of new blood vessels from existing ones, occurs in the skeletal muscle as an adaptive response to exercise that satisfies the increased requirement of this tissue for oxygen delivery and metabolic processes. Of the factors that have been identified to regulate this process, the endothelial cell mitogen vascular endothelial growth factor (VEGF) has been proposed to play a key role. The aim of this study was to measure the skeletal muscle VEGF mRNA content and arteriovenous protein balance across the working leg in response to a single bout of prolonged, submaximal exercise. Seven physically active males completed 3 h of two-legged kicking ergometry. Muscle biopsies were collected from the vastus lateralis muscle from both working legs, and blood samples were collected from one femoral artery and femoral vein before, during, and in recovery from exercise. We show that the exercise stimulus elicited a decrease in VEGF protein arteriovenous balance across the working leg (P = 0.007), and a ninefold elevation in skeletal muscle VEGF mRNA expression (P < 0.001). The changes in VEGF protein balance and mRNA content were most pronounced 1 h after the cessation of exercise. In conclusion, these findings demonstrate that submaximal exercise, suitable for humans with low CV fitness, induces a decrease in VEGF arteriovenous balance that is likely to be of clinical significance in promoting angiogenic effects.

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to participate in this investigation. Before participation, each subject was medically examined by a physician, and a resting venous blood sample was taken for blood screening. Subjects signed a written consent form after receiving both a verbal and written outline of all procedures and potential risks associated with this study. Ethical approval for this investigation was obtained from the Ethical Committee of Copenhagen and Frederiksberg Communities, Denmark and performed according to the Declaration of Helsinki.

Experimental Protocol

Determination of peak workload. All exercise was performed using the two-legged knee extension “kicking” exercise model. Subjects performed incremental, exhaustive exercise to obtain individual maximum workload (Wmax). After a 5-min warmup at 60 W, subjects continued kicking at 60 extensions/min until volitional exhaustion. Workload was increased by 10 W every 2 min.

Experimental trial. Subjects reported to the (exercise) laboratory at 8 AM in a fasted state having undertaken no vigorous exercise for at least 12 h. Each subject changed into appropriate experimental attire and lay in a supine position for the placement of an indwelling catheter into the femoral artery and femoral vein of one exercising leg under local anesthesia (lidocaine, 20 mg/ml; SAD, Denmark). The femoral artery catheter was inserted 2–5 cm below the inguinal ligament and advanced 5–10 cm proximally. The femoral vein catheter was inserted ~2 cm below the inguinal ligament and advanced ~5 cm distally to avoid contamination from blood draining from the lower abdomen and saphenous vein. Subjects performed 3 h of two-legged knee extension exercise at 50% of peak workload (W50%). The rate of extension was maintained at 60 extensions/min until volitional exhaustion. Workload was increased by 10 W every 2 min.

Blood samples. Blood samples from the femoral vein and femoral artery were collected before exercise (Pre); after 0.5 h (Ex0.5), 1 h (Ex1), 2 h (Ex2), and 3 h of exercise (Ex3); and 0.5 h (Rec0.5), 1 h (Rec1), and 3 h (Rec3) postexercise.

Muscle biopsies. Muscle biopsies of the vastus lateralis muscle were taken at Pre, Ex0.5, Ex1.5, Ex3, Rec1, Rec3, and Rec20 using the percutaneous needle method (Bergstrom, Sweden) with suction. Local anesthesia (lidocaine, 20 mg/ml; SAD, Denmark) was administered to the site before the biopsies. Immediately after each biopsy, muscle tissue was removed from the needle by using sterile tweezers, and the tissue was immediately frozen in liquid nitrogen.

Skeletal Muscle VEGF mRNA Expression

Total RNA was extracted using the acid-phenol method as described previously (3). Briefly, human skeletal muscle samples (~20–30 mg) were homogenized in 500 µl RNAzol B for 15–20 s, and then 50 µl chloroform-isooamyl alcohol were added to homogenized samples, inverted, and placed on ice for 5 min. Samples were centrifuged at 13,000 rpm for 15 min at 4°C, and the upper aqueous layer was carefully pipetted off. An approximately equal volume of isopropanol (ice cold) and 1 µl of 20 mg/ml glycogen were added to the samples, inverted gently, and left for 1 h at -20°C. Samples were centrifuged at 13,000 rpm for 15 min at 4°C, and the top aqueous layer was gently removed. The remaining pellet was washed by adding 500 µl 75% ethanol (diluted in diethylpyrocarbonate water), gently flicking the tube to dislodge the pellet, and centrifuged at 8,000 rpm for 10 min at 4°C. The aqueous supernatant was removed to expose the RNA pellet, and centrifuged at 3,000 rpm for 5 min at 4°C. The RNA content of each sample was quantified spectrophotometrically using the method of Sambrook et al. (14). Samples (1 µg) were reverse transcribed using Taqman Reverse Transcription Reagents using random hexamer primers according to the manufacturer’s instructions (Applied Biosystems) and diluted 1:6 in DEPC-treated water.

VEGF mRNA expression was measured by using RT-PCR using real-time PCR (SDS 7700, Applied Biosystems). Primers and probes for amplification of the VEGF gene were constructed from human specific sequence data (NCBI, Bethesda, MD) using DNA analysis software (Primer Express, Applied Biosystems). The sequences used to amplify VEGF are the following: forward primer 5'-CTT GCT GCT CTA CCT CCA CCA T-3', reverse primer 5'-ATG ATG CTG CCC TCC TCC TTC T-3', and TaqMan probe 5'-AAG TGG TCC CAG GCT GCA CCC A-3'. The probe was 5'-FAM and 3'-TAMRA labeled. A blast search of the amplified sequence showed only homology with the target gene. The concentrations of primers and probe were optimized for use in the PCR reaction, the amplification efficiency was determined, and similar amplification efficiency of the target gene and the endogenous control was verified. Samples were analyzed in triplicate using 2.2 µl of the diluted sample in a total volume of 25 µl reaction mixture consisting of Taqman Universal Mastermix (Applied Biosystems) containing AmpliTaq Gold DNA polymerase, AmpErase uracil-DNA glycosylase, dNTPs with dUTP, carboxy-X-rhodamine as passive reference and buffer components, 0.177 µg each of forward and reverse primers, 0.36-µg probe, and water. The housekeeping gene 18S was measured using a 5'-VIC and 3'-6-carboxy-tetramethyl-rhodamine-labeled predeveloped assay reagent (Applied Biosystems) in all samples in the same PCR run. A two-step PCR run was performed using the general profile: 50°C for 2 min + 95°C for 10 min + [95°C for 15 s and 60°C for 1 min] × 40. A threshold cycle (Ct), where the fluorescence signal from the reporter dye reaches a given level was determined for each sample reflecting the amount of the specific mRNA in the sample. For each sample, the VEGF mRNA content was normalized to the 18S mRNA mRNA (given a ΔCt value). All samples from a given subject were expressed as fold changes relative to the prevale, which was set to 1, using the ΔΔCt method (Applied Biosystems).

Plasma VEGF Concentration and Arteriovenous Difference

Plasma VEGF concentration was measured in femoral arterial and venous blood samples at all sample time points using high-sensitivity ELISA (R&D Systems; Abingdon, UK) according to the manufacturers instructions. The calculation of VEGF arteriovenous balance was calculated by subtracting the femoral vein VEGF concentration from the femoral artery VEGF concentration.

Statistical Analysis

Statistical analysis was performed using SigmaStat (version 2.03). All data were normally distributed and were therefore expressed as means ± SE. A one-way analysis of variance (ANOVA) for repeated measures was
used to determine the effect of acute exercise on VEGF mRNA content and femoral plasma arteriovenous protein balance. The Student-Newman-Keuls test for pairwise multiple comparison procedure was performed to identify the source of any significant differences. At all times, \( P < 0.05 \) was used to indicate statistical significance.

RESULTS

Physiological and Performance Characteristics

All of the subjects completed the 3-h exercise protocol. Heart rate during exercise was 128 ± 2.5 beats/min, and \( W_{50\%} \) was 70 ± 1.43 W. Predicted \( \dot{V}O_2\text{peak} \) was 3.72 ± 0.08 ml·kg·min\(^{-1}\).

Effect of Acute Exercise on Skeletal Muscle VEGF mRNA

Acute exercise increased VEGF mRNA expression (ANOVA, \( P < 0.001 \)) (Fig. 1). At Ex1.5, VEGF mRNA had increased 4.5-fold from Pre, and remained elevated until Rec3. VEGF mRNA peaked 1 h after exercise (Rec1), increasing over ninefold from Pre levels, and this was significantly higher than all other time points.

Effect of Acute Exercise on VEGF Arteriovenous Balance

Acute exercise decreased VEGF arteriovenous balance (ANOVA, \( P < 0.007 \)) (Fig. 2C). From Pre to Ex0.5, arteriovenous balance decreased from 18.06 ± 10.6 to 2.72 ± 3.7 pg/ml. Despite a slight elevation at Ex1 (6.4 ± 5.2 pg/ml), VEGF arteriovenous balance continued to gradually decline throughout the exercise period (−3.45 ± 8.3 and −3.11 ± 10.45 pg/ml at Ex2 and Ex3, respectively) and during the first hour of recovery (−14.46 ± 6.7 and −28.85 ± 14.4 pg/ml at Rec0.5 and Rec1, respectively). VEGF arteriovenous balance at Rec1 was significantly different from Pre and Rec3. After 3 h of recovery (Rec3), VEGF had
started to return to basal levels (5.26 ± 2.7 pg/ml). The change in VEGF arteriovenous balance over time was predominantly due to an increase in femoral vein VEGF concentration throughout the experimental period (ANOVA, $P = 0.019$), with no change in femoral artery VEGF concentration (ANOVA, $P = 0.397$, Fig. 2, B and C, respectively).

**DISCUSSION**

The main finding of the present study was that exercise of low intensity and long duration induced a decrease in VEGF arteriovenous protein balance across the working leg. In addition, an analysis of the kinetics of VEGF gene expression showed that VEGF mRNA was elevated during exercise but peaked 1 h after the cessation of exercise.

To our knowledge, this is the first time that VEGF arteriovenous protein balance has been measured in the working leg in humans. We clearly show that arteriovenous balance decreases during exercise and in recovery, predominantly due to a combination of the maintenance of femoral arterial plasma VEGF concentration and an increase in femoral venous plasma VEGF concentration. The shift from positive to negative arteriovenous balance occurred concurrently to the elevation in VEGF mRNA expression (Ex1.5), and both the greatest decrease in arteriovenous balance and the greatest elevation in VEGF mRNA occurred at Rec1.

An elevation in VEGF mRNA in skeletal muscle may induce a subsequent increase in VEGF protein synthesis, which is released into the extracellular matrix (ECM) and to the circulation. However, it is unlikely in the current study that the decrease in VEGF arteriovenous balance can be entirely accounted for by an increase in protein synthesis. If this were the case, it might be expected that the elevation in VEGF protein release from the working leg might be preceded by an elevation in mRNA content in the muscle tissue rather than the change in both VEGF mRNA content and arteriovenous protein balance occurring at similar time points. This suggests that another mechanism, rather than skeletal muscle protein synthesis, might also contribute to the rapid shift in arteriovenous balance reported here.

An elevation in VEGF protein release from the working muscle tissue may have also contributed to the change in VEGF arteriovenous balance. There is an increase in VEGF protein release into culture media when they are subjected to local mechanical stress. In the current study, mechanical stress via contraction of the working muscle is likely to have induced VEGF protein release from myocytes into the ECM, and in addition to exerting local angiogenic effects, would account for the elevation in VEGF protein in the femoral vein.

In conclusion, prolonged, low-intensity exercise induced a rapid decrease in VEGF protein arteriovenous balance across the working leg. This change is likely due to both an increase in VEGF production by, and release from, myocytes to the ECM. This finding demonstrates that exercise modes suitable for humans with low fitness level induce changes in VEGF that is likely to be of clinical significance in angiogenesis.

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**REFERENCES**


