Human VEGF gene expression in skeletal muscle: effect of acute normoxic and hypoxic exercise


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Richardson, R. S., H. Wagner, S. R. D. Mudalier, R. Henry, E. A. Noyszewski, and P. D. Wagner. Human VEGF gene expression in skeletal muscle: effect of acute normoxic and hypoxic exercise. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H2247–H2252, 1999.—Vascular endothelial growth factor (VEGF) is involved in extracellular matrix changes and endothelial cell proliferation, both of which are prerequisites to new capillary growth. Angiogenesis is a vital adaptation to exercise training, and the exercise-induced reduction in intracellular PO2 has been proposed as a stimulus for this process. Thus we studied muscle cell PO2 [myoglobin (MbPO2)] during exercise in normoxia and in hypoxia (12% O2) and studied the mRNA levels of VEGF in six untrained subjects after a single bout of exercise by quantitative Northern analysis. Single-leg knee extension provided the acute exercise stimulus: a maximal test followed by 30 min at 50% of the peak work rate achieved in this graded test. Because peak work rate was not affected by hypoxia, the absolute and relative work rates were identical in hypoxia and normoxia. Three percutaneous needle biopsies were collected from the vastus lateralis muscle, one at rest and then the others at 1 h after exercise in normoxia or hypoxia. At rest (control), VEGF mRNA levels were very low (0.38 ± 0.04 VEGF/18S). After exercise in normoxia or hypoxia, VEGF mRNA levels were much greater (16.9 ± 6.7 or 7.1 ± 1.8 VEGF/18S, respectively). In contrast, there was no measurable basic fibroblast growth factor mRNA response to exercise at this 1-h postexercise time point. Magnetic resonance spectroscopy of myoglobin confirmed a reduction in MbPO2 in hypoxia (3.8 ± 0.3 mmHg) compared with normoxia (7.2 ± 0.6 mmHg) but failed to reveal a relationship between MbPO2 during exercise and VEGF expression. This VEGF mRNA increase in response to acute exercise supports the concept that VEGF is involved in exercise-induced skeletal muscle angiogenesis but questions the importance of a reduced cellular PO2 as a stimulus for this response.

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METHODS

Subjects. Six sedentary males, weighing 79.8 ± 2.9 kg (mean ± SD), ages 26.7 ± 1.9 yr, and with heights of 175.3 ± 3.2 cm, volunteered to participate in this study after health histories and physical examinations were completed and informed written consent was obtained according to the University of California, San Diego, Human Subjects Committee requirements. None of the subjects had performed endurance exercise on a regular basis before the study and, as indicated by the mean maximum O2 consumption (VO2max) measured during conventional cycle ergometer exercise (VO2max = 33.9 ± 1.8 ml·kg⁻¹·min⁻¹), they were appropriately classified as sedentary.

Exercise apparatus. The knee-extensor ergometer used to produce an acute exercise stimulus was designed to limit exercise to the quadriceps muscles of the left leg (21). Briefly, subjects were semirecumbent in an adjustable chair with a special ankle boot placed on the left leg that was connected by a bar to the ergometer (Fig. 1 in Ref. 22). Contraction of the quadriceps muscles caused the lower part of the leg to extend from an angle of 90° to 170°. Therefore, the lower leg traveled with an arc-shaped trajectory of ~80°. The momentum of the ergometer passively returned the relaxed leg to the start position, and, as a result, the quadriceps muscle was functionally isolated (20). During exercise, the contraction rate was maintained at 60 min⁻¹.

Acute exercise stimulus. One week after a brief familiarization period, subjects underwent an acute knee-extensor exercise bout with their left leg, consisting of a 5-min unloaded warm-up followed by a graded maximal exercise test and then by 30 min of knee-extensor exercise at 50% of the maximum work rate achieved. The graded exercise test required subjects to maintain each work rate for 60 s, after which the work rate was incremented by 5 W. The subjects continued until they were unable to maintain a cadence of 60 rpm for the entire minute.

Muscle biopsies. Approximately 1 h (50–70 min) after knee-extensor exercise was completed, a muscle biopsy was taken from the exercised leg (left) and the rested leg (right). Several weeks later a muscle biopsy was taken from the right leg after acute knee-extensor exercise in hypoxia [fractional inspired O2 (FIO2) 0.12]. Because peak work rate was not affected by hypoxia, the absolute and relative work rates were identical in hypoxia and normoxia. Thus the exercise stimulus before the biopsy procedure was identical in either condition. The use of a single resting biopsy for either condition is supported by our observation that resting levels of human VEGF mRNA are consistently low with varying environmental/physiological changes preceding the sample, even with 8 wk of training (unpublished observation). All biopsies were taken from the vastus lateralis ~3.5 cm deep, 15 cm proximal to the knee, and slightly distal to the ventral midline of the muscle. The 5-mm diameter biopsy needle was attached to sterile tubing and a syringe to apply a negative pressure to assist in the muscle sample collection (12). Lidocaine (2%) was used as a local anesthetic and was infiltrated beyond the depth of the biopsy. The muscle samples from each biopsy were immediately frozen in liquid nitrogen and stored at −80°C. In summary, muscle biopsies were collected from muscles in three different states: 1) rested, 2) acutely exercised, and 3) acutely exercised in hypoxia.

RNA isolation and Northern analysis. Total cellular RNA was isolated from each muscle sample (muscle sample mass range 65–110 mg) by the method of Chomczynski and Sacchi (6). RNA preparations were quantitated by absorbance at 260 nm, and intactness was assessed by ethidium bromide staining after separation by electrophoresis in 6.6% formaldehyde-1% agarose gel. Fractionated RNA was transferred by Northern blot to Zeta probe membrane (Bio-Rad, Hercules, CA). RNA was cross-linked to the membrane by ultraviolet irradiation for 1 min and stored at 4°C. The blots were then probed with oligo-labeled [α-32P]deoxycytidine triphosphate cDNA probes, which had a specific activity of 1 × 10⁶ dpm/μg DNA (8). The human VEGF is a 0.93-kb cDNA fragment isolated from the Ecor I site of pUC-derived plasmid (17). The bFGF is a 1-kb Xho I fragment of human bFGF cDNA (16). Prehybridization and hybridization were performed in 50% formamide, 5 × SSC (20 × SSC is 0.3 M sodium chloride, 0.3 M sodium citrate), 10 × Denhardt’s solution (100 × Denhardt’s solution is 2% Ficoll, 2% polyvinyl pyrrolidone), 50 mM sodium phosphate (pH 6.5), 1% SDS, and 250 μg/ml salmon sperm DNA at 37 or 42°C. Blots were washed with 2 × SSC and 0.1% SDS at 50°C for the VEGF mRNA. Blots were exposed to XAR-5 X-ray film (Eastman Kodak, New Haven, CT) with the use of a Cronex Lightning Plus screen at −70°C. Autoradiographs were quantitated by densitometry (Media Cybernetics, Silver Spring, MD). Each blot was subsequently reprobed (after prior complexes were stripped) with a cDNA specific for 18S ribosomal RNA, and this signal was used to normalize the mRNA signal for minor variations in lane loading. All samples from a single subject were run on the same gel, producing optimum conditions for quantitative analyses across conditions.

Determination of intracellular Po2. The same exercise paradigm was performed in a 2.0-Tesla Oxford imaging magnet (Fig. 1 in Ref. 22). Spectra were collected from the muscle region below the 7-cm-diameter surface coil that was...
double-tuned to proton (85.45 MHz) and phosphorus (34.59 MHz) and placed over the rectus femoris portion of the quadriceps group (26) ~20–25 cm proximal to the knee. For these studies, this “sensitive region” was <100 cm³ of muscle, which isolated signal detection predominantly to the rectus femoris (1). Details of the theory behind O₂-sensitive myoglobin signals have been published previously (3, 22). Briefly, the heme iron exhibits O₂-dependent spin states that, in turn, influence nearby protons. The N-δ proton on proximal histidine F8, one of the ligands coordinated to the iron, is particularly sensitive to these changes. When O₂ is bound to the active site, the resonance of this proton is hidden beneath the dominant water signal. However, when myoglobin becomes deoxygenated, changes in the iron spin state shift this peak to a temperature-dependent position that is clearly distinct from all other resonances. Fractional deoxymyoglobin (fdeoxy-Mb) was determined by normalizing the signal areas to the average signal obtained during the 9th and 10th minutes of cuff ischemia at suprasystolic pressure (270 mmHg). Intramuscular O₂ depletes within 6–8 min of occlusion (28). Therefore, the plateaued signals obtained during the last 2 min of cuff occlusion represent complete deoxygenation of myoglobin and are used to estimate total myoglobin content within the muscle. Conversion to PO₂ values was then calculated from the O₂ binding curve for myoglobin as

\[ \text{PO}_2 = \frac{(\text{MbO}_2 \times \text{MbP}_{50})}{\text{fdeoxy-Mb}} \]

where \(\text{MbO}_2\) is the fraction of myoglobin that is oxygenated and \(\text{MbP}_{50}\) is the O₂ pressure at which 50% of the myoglobin binding sites are bound with O₂. The temperature-dependent myoglobin half-saturation (P₅₀) of 3.2 mmHg was used (25). On the basis of the previously published observation that intracellular PO₂ does not vary from submaximal efforts (50% of work rate maximum) (22), the data collected in several of the current subjects progressing toward their maximum work rate, and the inclusion of maximal exercise in the original exercise paradigm, intracellular PO₂ values in the magnet were measured during maximal knee-extensor exercise. Intracellular PO₂ data were only successfully collected on five of the six subjects because of a signal-to-noise problem.

Statistical analysis. The acute effect on resting muscle of either normoxic or hypoxic exercise was qualitatively assessed from the densitometry values for the VEGF and bFGF mRNA levels (normalized by the 18S values) using a repeated-measures ANOVA. Differences between groups were then identified using a Newman-Keuls post hoc analysis. Intracellular PO₂ and functional measurements of skeletal muscle work rate in hypoxia and normoxia were compared using paired t-tests. The relationship between intracellular PO₂ and VEGF mRNA was assessed with a regression analysis. Statistical significance was accepted if \(P < 0.05\).

RESULTS

Functional response to acute normoxic and hypoxic exercise. The average maximal knee-extensor work rate was not statistically different between exercise in normoxia (62 ± 5 W) and hypoxia (58 ± 6 W). Thus for these sedentary subjects both the absolute and relative intensities for the 30-min exercise stimulus in these two conditions were also not statistically different between normoxia and hypoxia.

VEGF response to acute normoxic and hypoxic exercise. As shown in Fig. 1, it is clear that at rest the VEGF mRNA levels of these sedentary subjects were very low. It is also evident that VEGF mRNA abundance in human skeletal muscle increased significantly 1 h after an acute small muscle mass exercise bout (Figs. 1 and 2). This VEGF mRNA increase is apparent whether the exercise was performed in normoxic or hypoxic conditions. In fact, Fig. 2 shows the observation that the VEGF mRNA responses to exercise in normoxia or hypoxia were significantly increased compared with resting control values, but the exercise values were not statistically different from each other. Figure 3 shows that no significant relationship exists between intracellular PO₂ during exercise and VEGF mRNA abundance after exercise.
bFGF response to acute normoxic and hypoxic exercise. Significant levels of bFGF mRNA were seen in the control condition (rested skeletal muscle = 0.9 ± 0.07 bFGF/18S arbitrary units; Fig. 1). Acute exercise in normoxia or hypoxia did not statistically alter the baseline values recorded in this skeletal muscle (0.8 ± 0.7 and 1.2 ± 0.2 bFGF/18S arbitrary units, respectively; Fig. 1). Thus, in contrast to the VEGF mRNA response, the bFGF mRNA response to exercise was not measurably different from control values (Fig. 1).

Intracellular PO2 response to acute normoxic and hypoxic exercise. The use of myoglobin as an endogenous probe for intracellular PO2 is limited by the fact that when myoglobin is bound to O2, it is not visible by magnetic resonance spectroscopy. However, on the basis of our previous observations, a P50 of 3.2 mmHg, and the signal-to-noise ratio in the present study, a myoglobin signal of ~20% of the maximal cuff signal should be detectable. Because there was no such signal at rest in these subjects, we can conclude that the intracellular PO2 at rest was >13 mmHg. Thus intracellular PO2 was reduced from resting levels during exercise in either normoxia or hypoxia. Intracellular PO2 during exercise in these subjects was significantly lower during exercise in hypoxia (3.8 ± 0.3 mmHg) than during exercise in normoxia (7.2 ± 0.6 mmHg). The relationship between individual subject VEGF mRNA response to exercise and the intracellular PO2 recorded at maximal exercise is shown in Fig. 3 (note that intracellular PO2 was measured in only 5 subjects). There was no relationship between intracellular PO2 measured during exercise and the level of VEGF mRNA after exercise (Fig. 3).

DISCUSSION

The principal finding of this study is that VEGF mRNA in untrained human skeletal muscle was substantially increased 1 h after a single acute exercise bout (Fig. 1). Because VEGF functions as a direct angiogenic factor with a high specificity for vascular endothelial cells (17), these findings are in line with the theory that VEGF is involved in the well-documented formation of new blood vessels within human skeletal muscles in response to exercise (5, 11, 30). This is an essential adaptive response in skeletal muscle to repeated exercise (i.e., training), resulting in an increase in the number of capillaries per muscle fiber that enhance O2 transport conductance between the microcirculation and mitochondria (2). Interestingly, the present data reveal a fall in intracellular PO2 from rest (>13 mmHg) to exercise in normoxia (7.2 ± 0.6 mmHg), but an even greater fall in intracellular PO2 in hypoxic exercise (3.8 ± 0.3 mmHg) did not demonstrate an additive effect on VEGF mRNA abundance. Perhaps this observation can be reconciled with the concept that hypoxia is a stimulus for angiogenesis by the existence of an intracellular PO2 “threshold” beyond which no greater angiogenic stimulus is produced. Exercise in normoxia appears to achieve this threshold.

VEGF response to hypoxic exercise. As recognized above, VEGF mRNA increased significantly after exercise in either hypoxia or normoxia, but the response was not significantly different or correlated with intracellular PO2 (Figs. 1–3). This disproves our hypothesis of an inverse relationship between intracellular PO2 and VEGF mRNA level and disagrees with the findings of Breen et al. (4), who recorded a doubling of the VEGF message in rats exercised in hypoxia compared with that in rats exercised in normoxia.

On the basis of both previous work in humans demonstrating that intracellular PO2 is low in normoxic exercise and even lower in hypoxic exercise (22) and a wealth of data from other systems indicating that VEGF is a hypoxia inducible gene (10, 18, 19), the reasonable inference was made that downward fluctuations of intracellular PO2 may be a signal for upregulating VEGF (4, 13). These data show that, as expected, intracellular PO2 fell significantly in hypoxia (Fig. 3). However, there was no relationship between intracellular PO2 and VEGF mRNA abundance within or between the two FIO2 conditions (Fig. 3). Two pertinent observations should be made here. First, in these untrained subjects, maximal work rate and VO2max were unaffected by this perturbation in intracellular PO2 (23), whereas in previous work with exercise-trained subjects, a reduction in intracellular PO2 resulted in a large and significant fall in muscle VO2max (22). This suggests that maximal exercise in trained subjects is O2 supply dependent, whereas O2 demand may play a larger role in the sedentary subjects studied here, who lack the enhanced mitochondrial volume of their trained counterparts. Second, the intracellular PO2 recorded in these untrained subjects during exercise in hypoxia is significantly higher than that recorded previously in exercise-trained subjects under the same conditions (2.1 ± 0.2 mmHg; Ref. 22). In fact, the hypoxic intracellular PO2 values recorded in these untrained subjects are even greater than the normoxic data recorded in an exercise-trained group previously studied with the same technique (3.1 ± 0.2 mmHg; Ref. 22). Both observations suggest that the importance of the recorded fall in intracellular PO2 in these sedentary subjects may have somewhat of a reduced physiological significance when compared with that of trained subjects. Thus for this population it is possible that the development of a larger mitochondrial capacity must precede an enhanced VEGF response to exercise in hypoxia because the relatively high intracellular PO2 (and apparently adequate O2 supply) does not yet signal the need for an elevated angiogenic response in these conditions. However, it is pertinent to again recognize that the present data reveal a significant reduction in intracellular PO2 from rest to exercise even in normoxia and that the reported VEGF mRNA increase in this condition may be due to a relative cellular hypoxia caused by the exercise. Hence, hypoxia per se cannot be ruled out as playing a role in the VEGF mRNA response of skeletal muscle to exercise.

bFGF response to acute exercise. In human skeletal muscle at rest there were significant levels of bFGF mRNA, contrasting with the low levels of VEGF mRNA in the same condition. Additionally, exercise in either normoxia or hypoxia did not lead to increased bFGF.
mRNA levels. Thus, in agreement with the findings and inferences of other work, we support the concept that bFGF may play a less significant role in the control of the angiogenic process than VEGF (4, 11, 15, 17). However, it should be recognized that this study is limited by the single muscle sample taken 1 h after exercise and that bFGF may increase beyond the measured time frame, although this was not evident in the previous exercise study in rats, which employed several sampling times after exercise (4).

Limitations to present study. There are several unavoidable limitations to our experimental design, including the fact that measurements of intracellular $P_O_2$ (dorsal area of quadriceps) were not recorded in the same anatomic site as the biopsies (vastus lateralis of the quadriceps). However, it has been documented that all quadriceps muscles are equally recruited in this exercise model (20). The $P_O_2$ measurements/biopsies were not taken at the same time, but the logistical complexity of this study precluded this. A single muscle sample limits conclusions, because the time course for VEGF mRNA may be affected by hypoxic exercise. Either a shortening or lengthening of the response time may lead to an erroneous conclusion, based on one sample. In the future, multiple biopsy samples need to be taken that will reveal both time-course issues and information beyond transcription to translation of angiogenic factors and receptors. Additionally, the unexpected trend toward a reduced VEGF mRNA response with decreasing $P_O_2$ (Fig. 3) may have achieved significance with a greater number of subjects in this study. However, such an observation is contrary to our final hypothesis and to the commonly accepted dogma that VEGF expression appears to be inversely related to $O_2$ availability.

In summary, we have documented a large and significant increase in VEGF mRNA in response to a single exercise bout in humans. This mRNA increase was not mirrored by bFGF. With the documented role of VEGF in the angiogenic process, these data suggest that the increased abundance of VEGF mRNA after exercise may play a role in instigating this important skeletal muscle adaptation to exercise. The same exercise stimulus in hypoxia elevated VEGF mRNA abundance to a level similar to that in normoxic exercise, despite a significant reduction in intracellular $P_O_2$. This latter observation fails to support the theory that intracellular $P_O_2$ is directly related to the VEGF-mediated angiogenic response. However, as $P_O_2$ falls from rest to exercise, this drop may achieve an intracellular $P_O_2$ threshold that stimulates angiogenesis.

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