Induction and maintenance of increased VEGF protein by chronic motor nerve stimulation in skeletal muscle

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Annex, Brian H., Carol E. Torgan, Pengnian Lin, Doris A. Taylor, Michael A. Thompson, Kevin G. Peters, and William E. Kraus. Induction and maintenance of increased VEGF protein by chronic motor nerve stimulation in skeletal muscle. Am. J. Physiol. 274 (Heart Circ. Physiol. 43): H860–H867, 1998.—Vascular endothelial growth factor (VEGF) causes endothelial cell proliferation in vitro and angiogenesis in vivo. Glycolytic skeletal muscles have a lower capillary density than oxidative muscles but can increase their capillary density and convert to a more oxidative phenotype when subject to chronic motor nerve stimulation (CMNS). We used Western analysis and immunohistochemical techniques to examine VEGF protein in a rabbit CMNS model of glycolytic skeletal muscle and in muscles with innate glycolytic versus oxidative phenotypes. VEGF protein per gram of total protein was increased in stimulated vs. control muscles 2.9 ± 1.0, 3.6 ± 1.3, 3.1 ± 0.5, 4.4 ± 1.6, and 2.7 ± 0.3 times after 3 (n = 4), 5 (n = 2), 10 (n = 3), 21 (n = 3), and 56 (n = 2) days, respectively. VEGF protein was increased 3.1 ± 0.5 times (P < 0.005) before (3, 5, and 10 days) and remained elevated 3.7 ± 1.0 times (P < 0.05) after (21 and 56 days) the transition to an oxidative phenotype. By immunohistochemistry, VEGF protein was found primarily in the matrix between stimulated muscle fibers but not in the myocytes. In addition, VEGF protein was consistently lower in innate glycolytic compared with oxidative muscles. These findings suggest that VEGF plays a role in the alteration and maintenance of vascular density in mammalian skeletal muscles.

angiogenesis; growth factor; vascularity; exercise; endurance training

Mammalian striated skeletal muscles are subject to markedly different functional demands. Skeletal muscles that are tonically active are composed of predominantly slow-twitch (type I) fibers and have an oxidative phenotype (19, 23). In contrast, skeletal muscles that are phasically active are composed of predominantly fast-twitch (type II) fibers and have a glycolytic phenotype (19, 23). Importantly, skeletal muscles can modulate their phenotype in response to changes in functional demand. This marked plasticity of phenotype is a unique feature displayed by mammalian skeletal muscle and occurs because of changes in contractile protein elements, proteins of oxidative metabolism, and local blood delivery (10, 11, 19, 21, 23, 26).

When compared with glycolytic muscle, oxidative muscles contain different subtypes of contractile proteins and greater amounts of proteins of oxidative phosphorylation (19, 21, 23). Oxidative (red) skeletal muscles also have a greater capillary density than glycolytic (white) muscles, and the capillary density in cardiac muscle may be as much as 10 times greater than glycolytic muscles (14). The capillary density in skeletal muscle, however, is not fixed but can be increased by chronic motor nerve stimulation (CMNS) or exercise conditioning (13, 14, 23, 26). In response to exercise training, the proliferation of blood vessels in exercised muscle fibers results in an increase in the number of capillaries per muscle fiber and capillaries per squared millimeter of muscle tissue (23). Moreover, after exercise conditioning the increases in the number of capillaries per muscle fiber parallel the improvements seen in the oxidative capacity of the muscle (16). Similarly, although animal models vary with the species studied and the stimulation protocol used, CMNS of glycolytic skeletal muscle causes a twofold increase in the capillary density in the target muscle, and the growth of blood vessels (i.e., angiogenesis) temporally precedes changes in oxidative proteins or the contractile protein composition (for review, see Ref. 13).

Angiogenesis is the growth and proliferation of new blood vessels and plays an important role in normal growth and development (6, 8). In fully developed organs, angiogenesis is important for tissue regeneration and remodeling (8). Because both exercise conditioning and CMNS result in increases in capillary density in skeletal muscles, angiogenesis appears to play a role in the adaptive response of mammalian skeletal muscle to increases in contractile work. The factor(s) that regulate angiogenesis in skeletal muscle, however, are poorly understood.

Vascular endothelial growth factor (VEGF) is a 46-kDa heparin-binding, homodimeric glycoprotein (6, 25). VEGF is an angiogenic growth factor that displays unique endothelial cell specificity mediated through two endothelial cell receptor tyrosine kinases (7, 8). Studies have implicated VEGF in tumor angiogenesis (22) and in the angiogenic response induced by myocardial ischemia (10). Two reports in rodent models have shown that VEGF mRNA, measured by Northern analysis of total muscle RNA, is increased after CMNS (9) and treadmill exercise (3a). However, changes in mRNA are not necessarily associated with alterations in protein levels, and VEGF is a secreted protein that may have effects distant from where it is produced (6–8).

Because no reports have examined VEGF protein in skeletal muscles, we used Western analysis and immunohistochemical techniques to quantify and localize VEGF protein in electrically paced versus control glycolytic skeletal muscle in a rabbit model of CMNS (19, 28, 29). On the basis of previous reports using this model, we examined time points before (3, 5, and 10 days of...
**METHODS**

Electrical stimulation protocols and tissue procurement. We implanted pulse generators in adult New Zealand White rabbits using methods previously described (27–29). Briefly, electrodes from miniature pulse generators were anchored in apposition to the common peroneal nerve, which innervates the fast-twitch muscle [tibialis anterior (TA), extensor digitorum longus (EDL), and peroneus longus] of the rabbit hindlimb. One hindlimb underwent continuous (24 h) pacing at 6–10 Hz for 3 (n = 4), 5 (n = 2), 10 (n = 3), 21 (n = 3), or 56 (n = 2) days. The same muscle from the contralateral unstimulated limb of each animal served as the control. The time points of 3, 5, and 10 days of CMNS were used as time points after the conversion to an oxidative phenotype (19, 28, 29). The time points of 21 and 56 days of CMNS were used as time points after the conversion to an oxidative phenotype (19, 28, 29). Under sterile conditions, muscles were removed from anesthetized animals, rinsed in sterile saline, and stored at −70°C as described (28, 29).

Samples from New Zealand White rabbit striated skeletal muscle with differing innate fiber-type compositions were also studied, including cardiac, oxidative, or slow-twitch type I (soleus and semitendinosus) and glycolytic or fast-twitch type II (plantaris and erector spinae) muscles. Samples of liver, spleen, skin, and kidney were used as negative controls for the Western analysis of myoglobin protein. External jugular veins were obtained and used as positive controls to verify the presence of VEGF protein and endothelial cells by immunohistochemistry studies. All procedures involving these animals conformed with the guidelines for use of laboratory animals published by the United States Department of Health and Human Services as approved by the Duke University Animal Use Committee.

Immunohistochemistry. VEGF protein in muscle samples was localized by immunohistochemistry, using modifications of methods previously described (2). Part of each frozen tissue sample was used for protein extraction, and the adjacent part was thawed in 30% sucrose-phosphate-buffered saline solution (PBS), placed in cross section at optimal cutting temperature (Miles Pharmaceuticals, West Haven, CT), and snap frozen in liquid nitrogen. Frozen sections (6 µm) were made in a cryostat on microscope slides (Superfrost Plus, Fisher Scientific, Pittsburgh, PA). Slides were allowed to come to room temperature, placed in ice-cold acetone for 2 min, and placed in PBS for 5 min. Blocking solution (10% horse serum in PBS) was applied for 1 h at room temperature. A murine anti-human VEGF antibody (clone 26503.11, Sigma Chemical, St. Louis, MO) was diluted (1:250) in blocking solution and applied to tissue sections for 1 h (7). Incubation with the primary antibody was followed by sequential incubation with a biotinylated anti-mouse immunoglobulin G (IgG) and ABC reagent, according to the manufacturer’s specifications (Vectastain ABC kit, Vector Laboratories, Burlingame, CA). Levanisole was added to block endogenous alkaline phosphatase activity, and immune complexes were localized using the chromogen alkaline phosphatase substrate Vector Red (Vector Laboratories). The sections were counterstained with hematoxylin, dehydrated, and mounted with Permount (Fisher Scientific). In this method, the antigen appears red and the nuclei are blue. To identify endothelial cells in serial sections, a murine monoclonal antibody (provided by K. G. Peters) raised against the soluble extracellular domain of the human tie-2 (tek) protein was diluted (1:100) in blocking solution. Tek is a receptor tyrosine kinase expressed exclusively in endothelial cells (5). In histological sections of rabbit external jugular vein (Fig. 1A) and carotid artery (not shown), this antibody reproducibly stained rabbit endothelium. For each muscle sample, a serial section was incubated with a non-sense murine IgG monoclonal antibody to serve as a negative control (2).

Protein extraction. Muscle samples (400–500 mg) were weighed, pulverized in liquid N2, and homogenized in 3 ml of 10 mM Tris(hydroxymethyl)aminomethane (Tris) (pH 7.4) and 100 mM NaCl, using a Brinkmann Polytron (Westbury, NY). The suspension was then centrifuged twice at 8,000 g at 4°C for 15 min. The protein content of the supernate was determined by the Bradford assay (3).

Western analysis. In preliminary studies, Western analysis of total muscle protein extracts (20 µg) was found to be unsuitable for a quantitative assessment of VEGF protein levels due to a low detection signal. Therefore, 200 µg of total muscle protein lysates were diluted to a total volume of 1 ml using 10 mM Tris (pH 7.4) and 100 mM NaCl; 25 µl of equilibrated heparin-agarose beads (Sigma Chemical) were added and the mixture was rocked at 4°C for 1 h, as previously described (7, 25). The beads were pelleted at 5,000 g for 1 min, washed in 0.4 M NaCl and 20 mM Tris (pH 7.4), and recentrifuged at 5,000 g for 1 min; the supernatant was removed and discarded. Next, 15 µl of 2× gel-loading buffer [20% glycerol, 100 mM Tris·HCl (pH 6.8), 4% sodium dodecyl sulfate, and 0.2% bromphenol blue] were added to the bead slurry. The VEGF was displaced from the beads by boiling the sample for 10 min. Samples were loaded onto an 8% sodium dodecyl sulfate-polyacrylamide gel and run at 150–200 V for 1 h. Proteins were transferred to nitrocellulose paper by electrophoresis. Blots were blocked in 5% nonfat dried milk, incubated with primary antibody, washed in TBST and incubated with a goat anti-mouse IgG horseradish peroxidase-conjugated antibody (Promega, Madison, WI) diluted 1:5,000 in TBST. An immunoreactive band at ~46 kDa was detected by chemiluminescence using enhanced chemiluminescence substrate (Amersham, Arlington Heights, IL) and X-ray film (X-AR; Kodak, Rochester, NY). Because the VEGF antibody was raised against the entire VEGF protein, not a polypeptide, recombinant human VEGF (R & D Systems, Minneapolis, MN) was preincubated at a concentration of 1 µg/ml with the VEGF antibody to confirm the specificity of the immunoreactive signal. To ensure that the binding of VEGF to heparin-agarose beads was complete and not saturable, 100, 200, and 400 µg of protein lysates from stimulated muscle were loaded onto heparin-agarose beads to confirm that corresponding increases in VEGF protein signal were present.

VEGF in stimulated vs. control muscle. Control and stimulated EDL muscles were examined after 3 (n = 4), 5 (n = 2), 10 (n = 3), 21 (n = 3), and 56 days (n = 2) of CMNS. Control and stimulated muscle samples from each animal were always handled in parallel and run on adjacent lanes in the same gel.
VEGF in striated skeletal muscles of differing fiber-type compositions. We analyzed a series of muscles with different innate fiber-type compositions based on previously published contractile protein isoform distributions (1, 4, 24). Plantaris, erector spinae, and TA muscle have been shown to be composed of predominantly (>90%) type IIa and IIb fibers, whereas soleus and semitendinosus are predominantly (>90%) type I fibers. For these analyses, equal (200 µg) protein aliquots were loaded onto heparin-agarose beads and assayed for VEGF, as described above. The protein fraction that did not adhere to the heparin-agarose beads was subject to electrophoresis and assayed for myoglobin protein by Western analysis using a murine monoclonal antibody raised against human myoglobin at a dilution of 1:500 (MG-1 Biogenex, San Roman, CA). In preliminary studies, this antibody recognized a single band (~17 kDa) exclusively in rabbit cardiac and striated skeletal muscle. In nonmuscle tissues (liver, spleen, skin, aorta, and kidney) that contained a 20-times excess of protein, no band was detected (data not shown).

Densitometry and statistical analysis. For the quantitative VEGF analysis, film was always exposed for a period of time that ensured that all signals were within the linear range of the detection of the film. Protein loads that yielded VEGF signals which bracketed the target signals were analyzed to ensure that all detection steps yielded data within the linear range of detection for the assay. The VEGF band density was quantified using an LKB Bromma Ultrascan XL Laser Densitometer. Protein from the fraction that did not bind to heparin-agarose beads was examined for the presence of residual VEGF protein and none was detected. Similarly, the protein eluted from the heparin-agarose beads was tested for the presence of residual myoglobin protein and none was detected.

Statistical comparisons of differences between the several-fold changes in VEGF protein for the stimulated and unstimulated limbs from each animal were assessed by a two-tailed Student’s t-test for paired variables. The ratio for all values are expressed as means ± SE. Differences among the several-fold changes in VEGF protein at the different time points were assessed by a two-way analysis of variance (treatment × time). A level of P < 0.05 was set for significance for all tests.

RESULTS

Immunohistochemistry. Immunohistochemistry was used to localize endothelial cells and VEGF protein in rabbit tissues (Fig. 1). In unstimulated EDL muscle, VEGF protein was present at low levels between muscle bundles (Figs. 1E and 2B). After 3 days of CMNS, there were more endothelial cells between the muscle bundles (Fig. 2, C vs. A), with an increase in VEGF immunostaining (Fig. 2, D vs. B). The increase in VEGF protein in the muscles after 3 days of CMNS was primarily located in the matrix between the muscle bundles. After 21 days of CMNS, there were still more endothelial cells per muscle fiber (Fig. 2, E vs. A), with a sustained increase in VEGF immunostaining (Fig. 2, F vs. B), in the stimulated vs. control EDL muscle. The increase in VEGF protein in the 21-day stimulated EDL muscle could be accounted for primarily by protein in the matrix between the muscle bundles but was also pres-
ent in nonendothelial and endothelial cells between the muscle bundles. The myocytes did not demonstrate VEGF immunostaining. If CMNS resulted in an increase in the diameter of the muscle fibers, then any increase in the number of endothelial cells per muscle fiber might result in no difference in the endothelial cell density in the muscle. However, after 21 days of CMNS, the muscle fiber diameter was not bigger and indeed appeared to be smaller than in the control muscles (note that the same magnification was used in all panels in Fig. 2). This finding is similar to other reports (18).

Quantitative assessment of effects of CMNS on VEGF protein. Western analysis was used to quantify changes in VEGF protein at time points ranging from 3 to 56 days following the initiation of CMNS. In most muscle samples, VEGF was detected as an immunoreactive band at ~46 kDa; however, in 3-day stimulated muscle, VEGF protein was present as two bands of slightly different molecular weights (Fig. 3, top left, bottom middle, and bottom right panels). The specificity of the immunoreactive band for VEGF was confirmed by preincubation of the antibody with recombinant VEGF (Fig. 3, bottom left and middle panels; note the left panel had a longer exposure time than the middle panel but still no VEGF signal was detected).

In all samples VEGF protein per microgram of total muscle protein was increased in the stimulated vs. control muscles for each animal, and representative

Fig. 2. Serial sections of rabbit unstimulated EDL muscle (A and B) and EDL muscle after 3 days (C and D) or 21 days (E and F) of chronic motor nerve stimulations (CMNS). Rabbit endothelium is shown at left (A, C, and E) and VEGF protein at right (B, D, and F). Increase in VEGF protein in muscle after CMNS is primarily in matrix between myocytes, but in 21-day CMNS muscle some of the increase is found in endothelial cells (i.e., just below and to the right of panel letters). All panels are ×80 magnification.
examples are shown in Fig. 3. The means ± SE for the ratio of VEGF protein in the stimulated vs. control muscle were 2.9 ± 1.0, 3.6 ± 1.3, 3.1 ± 0.5, 4.4 ± 1.6, and 2.7 ± 0.3 at time points after 3 (n = 4), 5 (n = 2), 10 (n = 3), 21 (n = 3), and 56 (n = 2) days of CMNS (Fig. 4), respectively. Although the magnitude of the increase in VEGF protein was greatest at 21 days, there was no statistically significant difference among the different stimulation time points examined. At the time points (3, 5, and 10 days of CMNS) that preceded the transition to an oxidative phenotype, VEGF protein was increased 3.1 ± 0.5 times in the stimulated vs. control muscle (P < 0.005). At the time points (21 and 56 days of CMNS) that followed the transition to an oxidative phenotype, VEGF protein was increased 3.7 ± 1.0 times in the stimulated vs. control muscle (P < 0.05).

Ratio of VEGF: Myoglobin protein in different striated skeletal muscles. By examining the fraction of total muscle protein that bound to heparin-agarose beads for VEGF protein and the unbound fraction for myoglobin, we were able to simultaneously examine VEGF and myoglobin proteins, relative to total cellular proteins, in different skeletal muscles. As shown in Fig. 5, VEGF and myoglobin protein were both relatively low in glycolytic skeletal muscles (plantaris and TA) compared with oxidative skeletal muscles (soleus and semitendinosus). Similar results were obtained using erector spinae (>90% type II fibers) and cardiac muscle (data not shown). These findings demonstrate that across skeletal muscles with different fiber-type compositions, VEGF protein concentrations were proportionate to myoglobin protein and thus appeared to correlate...
with the overall oxidative capacity of rabbit skeletal muscles.

**DISCUSSION**

CMNS of glycolytic skeletal muscle is a model frequently used to examine the changes in skeletal muscles during and after the transition from a glycolytic to an oxidative phenotype (19, 21, 23). Among other changes, CMNS results in a twofold increase in the capillary density in the target muscle fibers after 4 wk, but the proliferation of blood vessels in the target muscle is present as early as 2 days following the onset of CMNS (13, 14). Therefore, angiogenesis precedes the changes in oxidative proteins or the contractile protein composition in skeletal muscle in this model and appears to play an important role in the adaptive response of skeletal muscle to increases in contractile work.

Our study is the first to demonstrate that VEGF is increased at the protein level in muscle in response to CMNS, and this result provides strong support for the hypothesis that VEGF plays a role in angiogenesis in mammalian skeletal muscles. VEGF is a prototypic angiogenic growth factor that causes endothelial cell proliferation in vitro and is associated with angiogenesis in vivo (6–8). Using a rodent model, Breen et al. (3a) demonstrated that VEGF mRNA was increased after only 1 h of treadmill running. Hang et al. (9) demonstrated that VEGF mRNA was increased 6-fold after 4 days and 1.5-fold after 21 days in rat skeletal muscle subject to CMNS. This group noted that when the approximately 2-fold greater yield of mRNA per gram of tissue in the 21-day stimulated vs. control muscles was adjusted, the overall increase in VEGF mRNA was approximately 3-fold at 21 days. Changes in mRNA are not necessarily accompanied by changes in protein. Moreover, VEGF is a secreted protein that may have effects distant to where it is produced (6, 7). Our study demonstrates that VEGF protein is increased in whole muscle homogenates following CMNS. We further demonstrate that the increase in VEGF protein is predominantly in the matrix between myocytes (the areas where the increase in the endothelial cells per muscle fiber is found), not in the muscle fibers themselves.

Hypoxia is a potential mechanism for the increases in VEGF protein that occur with CMNS of glycolytic skeletal muscle (9). Indeed, VEGF mRNA levels are increased in ischemic areas of solid tumors as well as in ischemic regions of the myocardium, and VEGF appears to be a potential regulator for angiogenesis in these conditions (8, 10, 22). Although our data do not exclude hypoxia as the stimulus for the increase in VEGF protein that follow the onset of CMNS, the magnitude of the increase in VEGF protein was similar at all time points examined and was maintained even after 21 and 56 days. Hypoxia is not likely to account for the sustained increases in VEGF protein seen in our study. In addition, studies that have performed direct measurements of oxygen tension in skeletal muscle have failed to demonstrate a relationship between capillary density and oxygen tension, either at rest or after the onset of CMNS (15).

**Fig. 4.** Bar graph showing magnitude of severalfold increase in VEGF protein levels in stimulated vs. control extensor digitorum longus (EDL) muscles of New Zealand White rabbits at time points of 3 (n = 4), 5 (n = 2), 10 (n = 3), 21 (n = 3), and 56 days (n = 2) following the start of continuous CMNS. Magnitude of severalfold increase is illustrated along with mean (±) standard error measurement (error bar). There were no statistically significant differences among the severalfold increases at different time points.

**Fig. 5.** Western blots of 200 µg of skeletal muscle protein from New Zealand White rabbits. VEGF is detected in protein by incubating protein lysates with heparin-agarose beads, eluting with sample buffer, followed by electrophoresis through a 8% polyacrylamide gel. VEGF is localized as a band at ∼68 kDa. Unbound protein, 4 µg (vertical arrows) of original 200 µg, is subjected to electrophoresis in a 12% polyacrylamide gel. Myoglobin is detected by a band at ∼17 kDa. Position of MMM is shown.
Oxidative skeletal muscle is known to have a greater capillary density than glycolytic muscles (13, 14). In an examination of skeletal muscles with different innate metabolic demands and fiber-type compositions, we found that VEGF protein per microgram of total cellular protein was consistently greater in oxidative (type I) than glycolytic (type II) muscles. This finding was based on previously published characterizations of rabbit skeletal muscles fiber-type composition and the parallel examination of VEGF and myoglobin protein in the muscle samples. Therefore, our data not only further support a role for VEGF protein in the angiogenic response induced by CMNS in skeletal muscle but suggest that higher levels of VEGF protein are part of the phenotype of oxidative skeletal muscle. The reason for the higher levels of VEGF protein in oxidative versus glycolytic skeletal muscles is unknown, but these findings raise the possibility that higher levels of VEGF protein are required for the maintenance of the greater vascular density in oxidative versus glycolytic skeletal muscles.

Although results from our study further support a role for VEGF in the angiogenic response induced by CMNS, the regulation of angiogenesis in skeletal muscle that occurs from increases in functional demands (CMNS or exercise conditioning) is likely to be complex. Yamashita et al. (30) observed that muscle extracts from exercise-trained rodents were able to stimulate angiogenesis in vitro. Morrow et al. (20), using the same model of CMNS that we used in this present study, demonstrated that protein extracts from stimulated muscles promoted angiogenesis as determined by the ability to increase [3H]thymidine incorporation in NIH 3T3 cells in vitro. Morrow et al. (20) also observed that acidic and basic fibroblast growth factor proteins, two other angiogenic molecules, were increased after 21 days of CMNS. Therefore, the number of potential mediators of angiogenesis in skeletal muscle is likely to be large, and there may be multiple pathways that modulate vascular density, which appears to be an important and highly regulated component of the phenotype in mammalian striated skeletal muscle.

In conclusion, we observed that VEGF protein was increased following the onset of CMNS and that VEGF protein colocalized with angiogenesis in the stimulated muscle. In addition, we found that higher levels of VEGF protein are part of the phenotype of oxidative versus glycolytic skeletal muscle. These findings strongly suggest that VEGF plays a role in both the alteration and maintenance of vascular density in mammalian skeletal muscles.

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